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SEASONAL VARIATIONS IN THE RATE OF PASTURE REGROWTH AFTER GRAZING

by G. RAPPE

Vassmolösa (Sweden)

From 1919 to 1929 the author was the manager of a farm in Southern Sweden where he made two observations concerning seasonal variations in the growth of pastures. (I) That changes in yield occurred when the grazing schedule was altered from one year to another, so that periods of grazing and of "rest" occurred at other times than before, while there were no other known changes. In the following this phenomenon will be referred to as "the relative change in yield". (II) That at the beginning and middle of July there occurred, regularly and apparently independent of rainfall conditions, a period of marked scarcity in available pasture, evidently caused by weak plant growth during the middle and later parts of June. This latter phenomenon is here referred to as the "midsummer depression".

Utilizing material from 11 years' grass cut experiments (1933–45) *). An attempt was made to find out whether the two phenomena mentioned were more or less incidental to the author's personal experience or whether they occur regularly in pasture-farming.

The experiments mentioned here have confirmed the occurrence of the two phenomena, and they show moreover that the arrangement of grazing periods at different times not only has different effects on the total yield obtained, but also changes its distribution from early to late summer, it has also been found that the above phenomena are characteristic only of the grass ingredients of a pasture.

*) Experiments carried out by the Swedish Peat Cultivation Society and its successor, the Swedish Grassland and Peat Cultivation Society, the research work of which has since 1948 been taken over by the National Agronomy Experiment Station of Sweden.

A plausible explanation of these phenomena seems to be that during the season of vegetative activity the plants pass through a sequence of phases of internal development. It is assumed that plants will run through these various phases whether they are allowed to grow until they reach the flowering and seeding stages or whether they are subjected to repeated grazing. This sequence of phases is therefore supposed to have been consolidated genetically.

As far as I know no investigations specifically directed to these two phenomena have been made. Various authors concerned with other questions have, however, touched upon the problem of seasonal variations in the growth of pasture plants, both as regards individual species and mixed swards.

A work of fundamental importance is R. G. Stapledon's [12]) "The Seasonal Productivity of Herbage Grasses with Particular Reference to Different Systems of Cutting etc.", 1924, what discusses problems closely related to those outlined above. There are several publications of his assistants and successors (edited by the Welsh Plant Breeding Station and the Bureau of Pastures and Forage Crops) directed to similar subjects; cf. also the investigations of H. E. Woodman *et al.* [14]) on the seasonal variations in the nutritive value of pastures and G. Nilsson-Leissner's [5]) [6]) investigations regarding the influence of meteorological conditions on the growth of pasture plants as well as regarding the relative contribution of individual plant species to the total yields obtained, and the seasonal variations thus involved. G. Giöbel [2]) has studied the influence of nitrogen fertilization at different seasons, and E. G. Mulder [4]), too, has dealt with this question. M. L. t'Hart [3]) has made an investigation into the seasonal variation in the growth of grasses, and lastly, H. J. Frankena [1]) has on several occasions closely touched upon the questions investigated by me; but none of the above authors have made them the direct object of their studies.

Among publications dealing with rhythm and periodicity in plants in general, I wish to mention especially the excellent survey given by R. O. Whyte [13]), and also L. G. Romell's [11]) report upon this subject.

RELATIVE CHANGE IN YIELD

While the grass plants are passing through their successive phases in spring their sensitivity to cutting or grazing seems to be subject to considerable variations. If cutting is performed at an early stage of development it will influence subsequent growth in a different manner from cuttings carried out at a later stage; this remarkable fact was brought out very clearly by my experiments of 1943–45 (24 experiments in southern Central Sweden and 18 experiments in North Sweden) [10]). In every experiment a study was made of the different yields obtained and of their distribution in two different cutting series (= plot groups). In plot group I the first harvest in spring was taken about 14 days after the assumed beginning of growth, and the following harvests were taken with intervals of 28 days. In plot group II the first harvest in spring was taken about 28 days after the assumed beginning of growth, the following harvests likewise being taken at 28-day intervals. In the 24 Central Swedish experiments, the total yields (i.e. the sums of the yields obtained at the individual harvests) were always different for the two plot groups, while in the North Swedish experiments they were different in 13 out of the 18 cases. The degree of difference between the total yields as well as their differences of distribution are exemplified in Fig. 1 and in Figs. 3*a* and 5*a*. Corresponding weather data are given in Figs. 3*b* and 5*b*.

Figs. 1, 3*a* and 5*a* show (I) the average daily production, represented by the height of the rectangles, within each period between two harvests, and (II) the yields obtained at each harvest, represented by the area of the rectangles. The vertical line —·—·— marks the 21st of June i.e. the summer solstice. Among the vertical lines of the rectangles, the first at the left marks the date when growth is assumed to have started whereas the others indicate harvest dates which may be read off at the bottom of the diagrams. For plot group I the sides of the rectangles have been drawn as uninterrupted lines and for plot group II as interrupted lines. In principle, all the areas representing plot group I have been left white while all the areas representing plot group II have been striped. (Most often, however, striped areas and white areas will overlap, and in this case parts of the originally white areas will naturally appear as striped). An exception from this rule has been made for

the first harvesting period of plot group II where a part of the upper portion of the first rectangle has not been striped. This white area is of equal size as the striped area (surrounded by a dotted line) above the aforesaid rectangle and is to be replaced by that area. The reason why the diagrams have been arranged in this manner is given below:

As plot groups I and II both represent the same pasture (in each of four parallel plot pairs, the plot of group I adjoins that of group II) the following conclusion may be drawn: during the first part of the first harvesting period of group II (i.e. the first harvesting period of group I) the production of group II must have been the same as that of group I, experimental errors being disregarded. Thus, all the remaining amount of herbage obtained from plot group II during the entire first harvesting period of this group must have been produced during the second part of this period (i.e. the period between the first harvest of group I and the first harvest of group II).

In each diagram *a* is the area of the rectangles on the left of the midsummer line and *b* the area on the right of that line, i.e. *a* is the total yield before midsummer and *b* the total yield after midsummer. (In my earlier publication [10]) from which this experimental material is taken I have drawn curves on the basis of the rectangles, but these curves have been omitted here, and all the statements made from the figures shown in this article are based on the rectangle diagrams only.

In the following, the individual diagrams of Fig. 1 will be explained together with the diagrams of Figs. 3*a* and 5*a* which are similarly constructed.

Figs. 1*f* and 5*a* (1943) illustrate cases where the change from an early (plot group I) to a late (plot group II) first harvest has diminished the *a* yield but increased the *b* yield in such a manner that the total yield has remained virtually *unchanged.*

Fig. 1*e* illustrates a case where the change from an early to a late first harvest has led to an increase in *a* yield as well as *b* yield and has thus considerably *increased* the total yield.

Figs. 1*b* and 5*a* (1945) illustrate cases where the change from an early to a late first harvest has resulted in a definite *increase* of the total yield which increase, however, is almost exclusively concentrated in the *b* yield.

Figs. 1*d* and 3*a* (1945) illustrate cases where the change from an

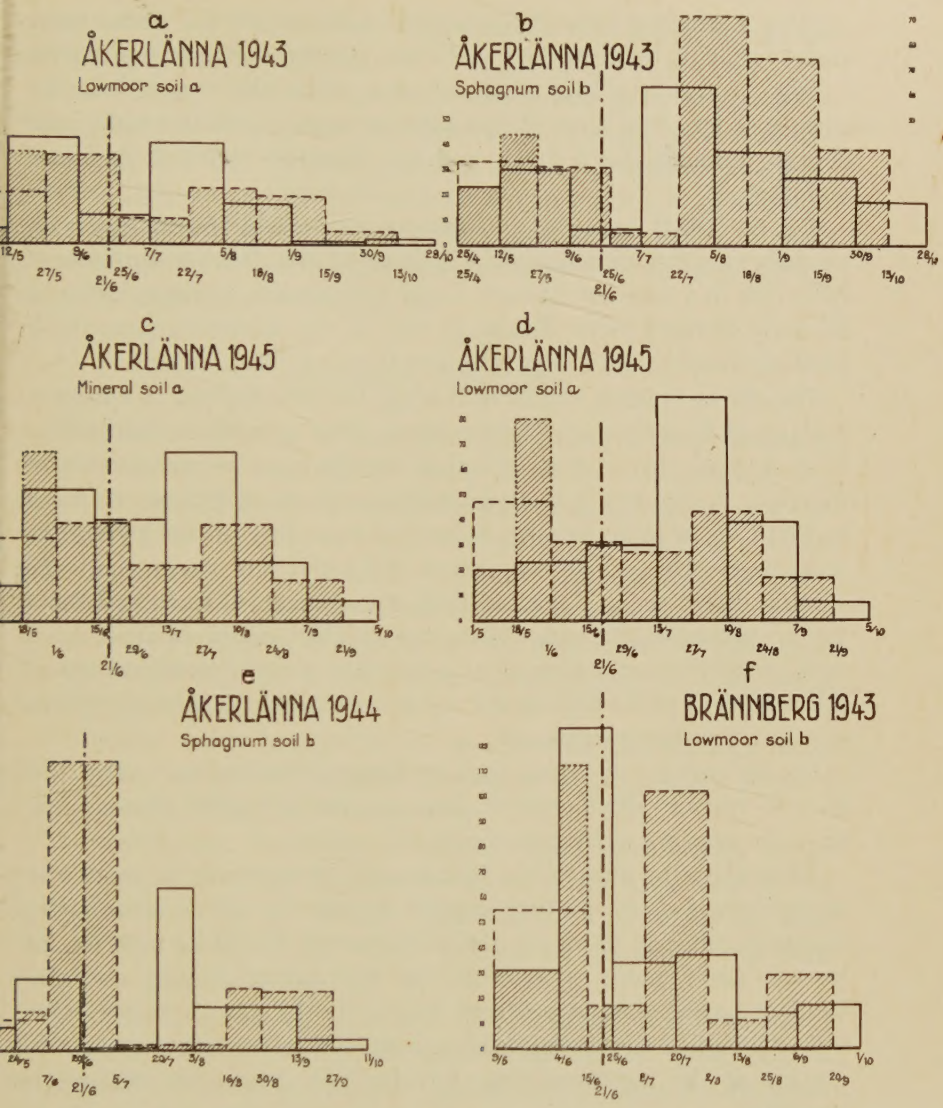


Fig. 1. The difference in total yields and in their distribution in plot groups I and II. The harvest dates of group I (early first harvests) are marked by uninterrupted vertical lines of the rectangles and those of group II (late first harvests) by interrupted vertical lines. The summer solstice is indicated by a —·—·— vertical line. All these dates are incerted under each vertical line, whereas the figures of the scale at the left of the diagram indicate the daily production as kg air-dried hay. Examples taken from *Åkerlänna* in the interior of southern Central Sweden and from *Brännberg* not far from the coast of North Sweden. The phosphate and potassium status was satisfactory in the Åkerlänna soils and good in the Brännberg soils. — 1*a*. *Åkerlänna 1943:* Pasture not fertilized with nitrogen on very weakly drained lowmoor soil. — 1*b*. *Åkerlänna 1943:* Pasture well fertilized with nitrogen on strongly drained Sphagnum soil. — 1*c*. *Åkerlänna 1945:* Pasture not fertilized with nitrogen on normally drained mineral soil. — 1*d*. *Åkerlänna 1945:* Pasture not fertilized with nitrogen on very weakly drained lowmoor soil. — 1*e*. *Åkerlänna 1944:* Pasture well fertilized with nitrogen on strongly drained Sphagnum soil. — 1*f*. *Brännberg 1943:* Pasture well fertilized with nitrogen on well drained lowmoor soil.

early to a late first harvest has increased the *a* yield but at the same time so sharply reduced the *b* yield that the total yield is *diminished.*

Figs. 1*c* and 3*a* (1943) illustrate cases where the change from an early to a late first harvest has without reducing the *a* yield considerably reduced the *b* yield, and has therefore reduced the total yield too.

Fig. 3*a* (1944) similarly illustrates a case where the change from an early to a late first harvest has resulted in a *decrease* of the total yield due to a decrease of both *a* and *b* yields but, mainly due to a decrease of the *b* yield; finally in Fig. 1*a* the *decrease* affects the *b* yield exclusively and a small increase is seen in the *a* yield.

The above examples show that when the first harvest in spring is postponed to a date about four weeks after growth is assumed to have started; the total yield will in certain cases be increased, and this may be due either to an approximately equal increase in the *a* and *b* yields or to an increase only, or almost only, in the *b* yield. In some cases the said postponement will not bring about any substantial difference in the total yield, but changes of the *a* and the *b* yields approximately compensate one another. Again, in other cases such postponement will lead to a decrease in total yield, always to the detriment of the *b* yield, the *a* yield remaining either more or less unaffected or being increased.

On the whole, it seems that in Central Sweden an early first harvest favours production in late summer (*b* yields) whereas the opposite appears to be true for North Sweden.

Evidently, the date of the first grazing in spring is of particular importance for the pasture supply during the whole season. By choosing suitable, dates for the first grazing it will be possible, at least to some extent, to influence the distribution of yield over the summer period; but in order to determine the most suitable dates a knowledge of the morphological condition of the various pasture grasses at the optimum time for the first grazing or cutting is essential.

It is quite conceivable that different species of pasture grasses may prove to have a different phase rhythm i.e. species may pass through their various phases at various times. If that were so, one might profit from such different rhythms, and avoid grazing during unsuitable periods, by providing a series of pastures some of which might consist mainly of grasses with one rhythm and others of

grasses with another rhythm. Experiments with single species are going on already (see below).

If on the other hand all grass species should prove to follow the same phase rhythm a somewhat similar effect might be obtained by providing, as far as this were possible within the scope of one farm, pasture grounds on different soils on which grasses would start to grow at different times in spring (different soil material, different ground water level or different irrigation conditions, different fertilizing e.g. nitrogen fertilizing etc. [9]).

MIDSUMMER DEPRESSION

There are assumed to be three main phases in the development of the perennial grasses during the season, one before midsummer, viz., the phase providing a preparation for the reproductional phase, one around the summer solstice, viz., the reproductional phase, and one after midsummer, viz., the phase providing a preparation for hibernation and the next year's reproduction. The vegetative growth of the grass plants is greatest during the first phase, and is rather good during the last phase, too, but is usually considerably reduced during the reproductional phase.

According to my hypothesis the grasses still pass through these phases when subjected to regular grazings or cuttings which prevent them from reaching the flowering stage.

The midsummer depresssion is supposed to be due mainly to the plants passing through this reproductional phase some time in the middle of the summer. During this period natural conditions may thus be less favourable to weight increase of the grass crop than during the periods before and after.

Figs. 2*a*, 2*b*, 3*a*, 3*b*, 4*a*, 4*b*, 5*a* and 5*b* have been taken from one of my earlier publications [10]). They are diagrams of some typical cutting series from natural pastures and of weather data for the years and places where the cutting series were obtained.

Fig. 2*a* represents harvests during five successive years from a very old pasture on Sphagnum soil (Flahult in the interior highland of South Sweden). Fig. 2*b* shows the weather diagrams for the same place and times. Similarly, Figs. 3*a* and 3*b* show harvests and weather data obtained at Åkerlänna in southern Central Sweden (three successive years, lowmoor soil); Figs. 4*a* and 4*b* show harvests and

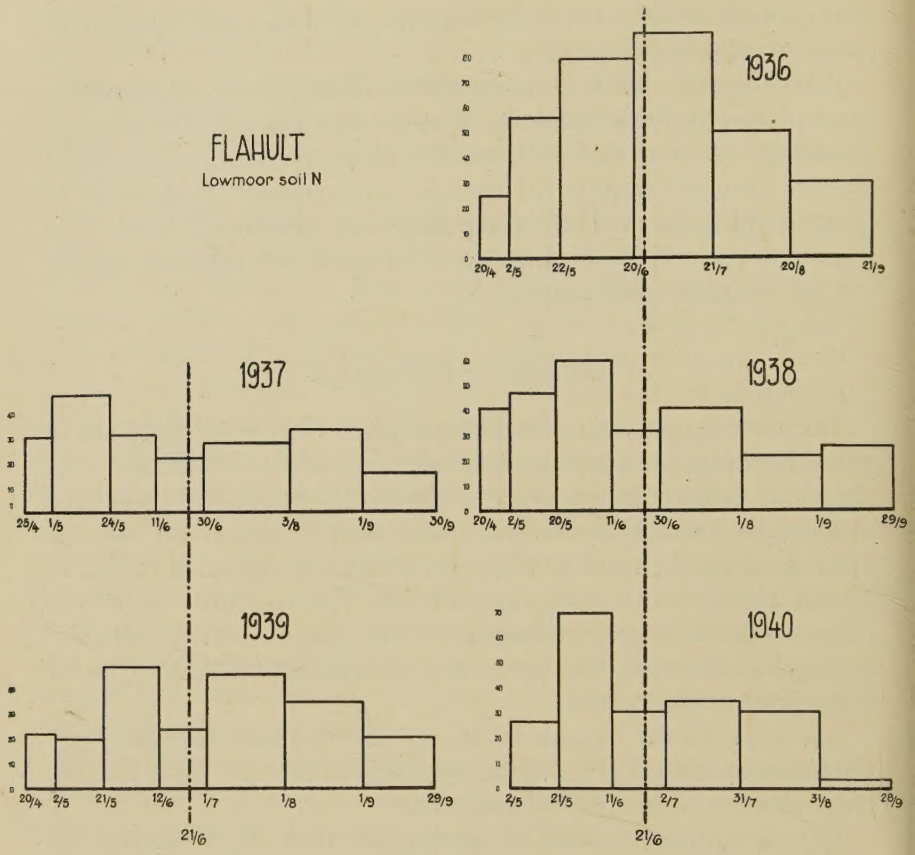
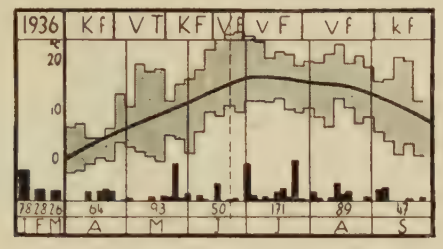
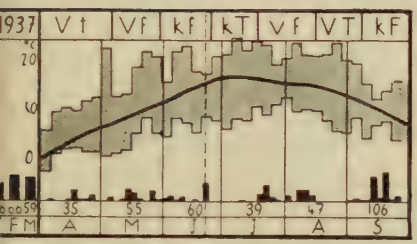


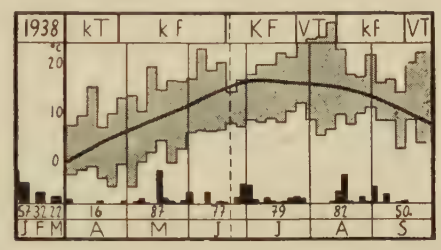
Fig. 2*a*. Simplex cutting series during five successive years at *Flahult* in the interior of South Sweden, 223 m above the sea level; nitrogen-fertilized pasture on well drained Sphagnum soil. Phosphate and potassium status good. Cf. the text under Fig. 1 and the text on page 311.

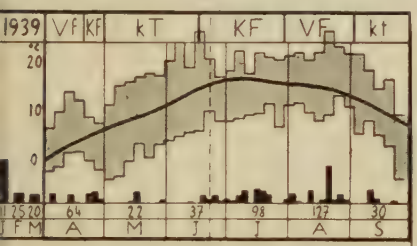
weather data obtained at Gisselås in the interior highland of northern Central Sweden (five successive years, lowmoor soil); Figs. 5*a* and 5*b* finally show harvests and weather data obtained at Brännberg in North Sweden (three successive years, lowmoor soil).

Figs. 3*a* and 5*a* show for each year two cutting series run in parallel. In all the series shown in Figs. 2–5 the pastures were fertilized with potassium and phosphoric acid early in spring; where nitrogen fertilizers were applied the treatments were equally distributed over the whole season so that equal amounts of nitrogen were supplied in spring and again after each harvest had been taken.









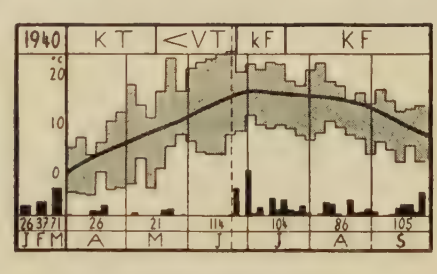


Fig. 2*b*. Weather diagrams for the five experimental years at *Flahult* (Fig. 2*a*). Letters at the top form the so-called "weather strip" in which

V = very warm
v = normal warmth.
k = somewhat sub-normal temperature.
K = markedly ,, ,,

} Subjectively estimated from the weather diagrams.

T = Very dry, less than 5 mm precipitation per week.
t = Dry, 5–10 mm precipitation per week.
f = Humid, 10–20 mm precipitation per week.
F = Very humid, more than 20 mm precipitation per week.

Curve shows mean temperature values calculated from the maximum and minimum temperatures of nine years, viz., the five experimental years + two years before and after. Around the curve, diagram of maximum and minimum temperatures during experimental period, given in 5-day periods. Below, black rectangle diagram indicating precipitation in mm (3-day periods for April–September; 1-month periods for the rest of the year). Letters at bottom indicate names of months.

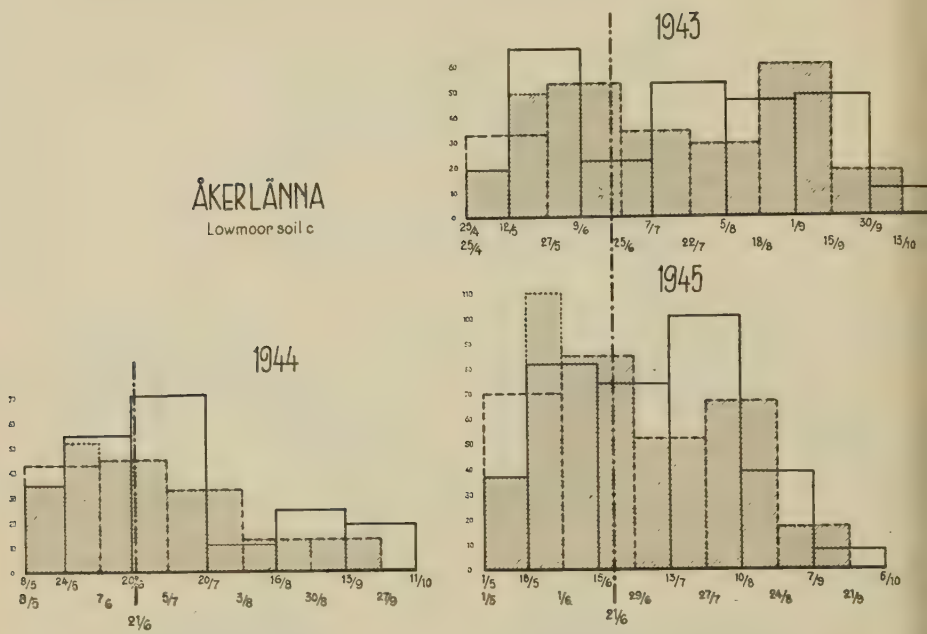


Fig. 3*a*. Double cutting series during three successive years at *Åkerlänna* in the interior of southern Central Sweden, around 35 m above the sea level; pasture fertilized abundantly with nitrogen on poorly drained lowmoor soil. Phosphate and potassium status satisfactory. Cf. the text under Fig. 1 and the text on page 311.

Fig. 2*a* shows 4 years (4 series) with midsummer depression and 1 year (1 series) without midsummer depression. Fig. 3*a* shows 2 years (4 series) with and 1 year (2 series without) midsummer depression. Fig. 4*a* shows 2 years (2 series) with and 3 years (3 series) without midsummer depression. In Fig. 5*a* there is one year in which one of the two parallel cutting series shows midsummer depression whereas the other does not, and 2 years where both series lack midsummer depression; we thus obtain 1 series with and 5 series without midsummer depression. The above data are summarized in the following table: (see page 320)

As appears from this table, the number of cutting series showing midsummer depression is greater in the South than in the North of Sweden. In the following, series with a depression around midsummer will be referred to as series of southern type, and series without such depression as series of northern type.

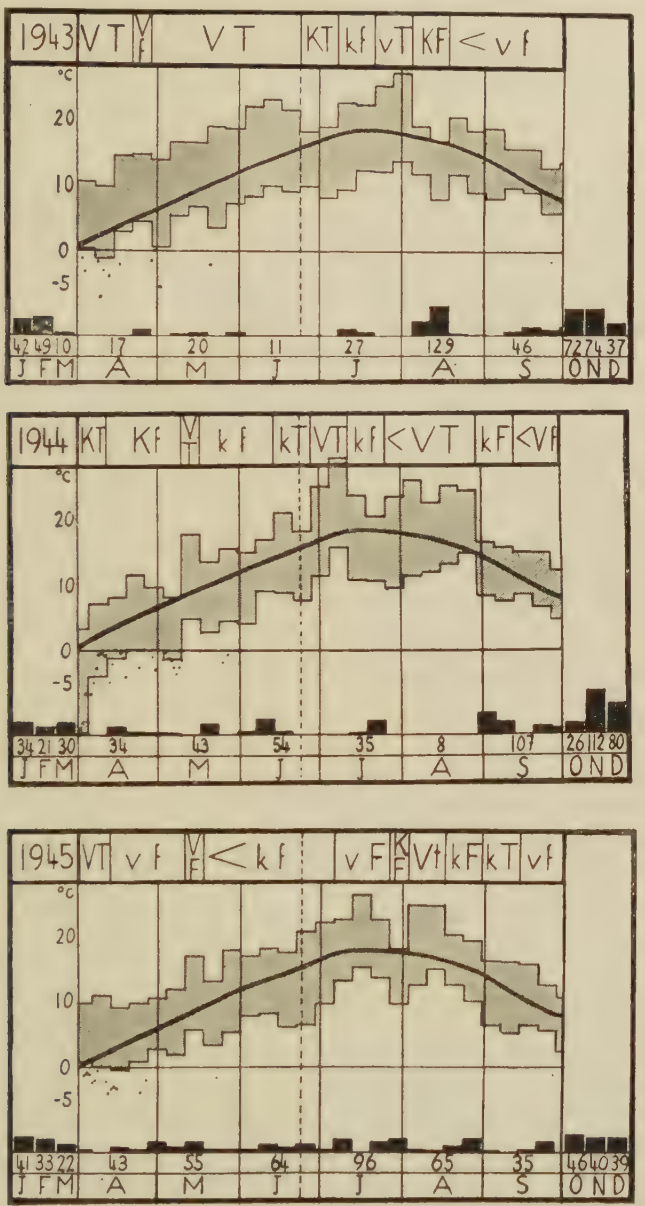


Fig. 3*b* .Weather diagrams for the three experimental years at Åkerlänna (Fig. 3*a*). Curve shows mean temperature values calculated from the maximum and minimum temperatures of seven years, viz., the three experimental years + two years before and after. Around the curve, diagram of maximum and minimum temperatures during experimental period, given in 7-day periods. Black dots under the temperature diagram mark out single days with minimum temperatures below 0°C. Black rectangle diagram below gives mm precipitation (7-day periods for April–September).

For further details see the text under Fig. 2*b*.

TABLE I

Numbers of diagrams obtained from experiments carried out during the years 1933–1945			
Geographical location	Diagrams with depressions in the middle of the summer	Diagrams without depressions in the middle of the summer	Total
The material as graphically represented here: Rectangle diagrams:			
South Sweden (Fig. 2*a*)	4	1	5
Southern Central Sweden (Fig. 3*a*) . .	4	2	6
Northern Central Sweden (Fig. 4*a*) .	2	3	5
North Sweden (Fig. 5*a*)	1	5	6
Total			22
The total material treated in my earlier publication (see above): Rectangle diagrams:			
South Sweden.	80	52	132
Southern Central Sweden	31	49	80
Northern Central Sweden	4	16	20
North Sweden	8	28	36
Total			268
Curves drawn on the basis of the rectangle diagrams:			
South Sweden.	95	37	132
Southern Central Sweden	50	30	80
Northern Central Sweden	6	14	20
North Sweden	10	26	36
Total			268

Possible Ways of Compensating Midsummer Depression.

It has been suggested that the economic difficulty arising from poor pasture supply during the time of midsummer depression might at least in part be overcome by artificial means, such as nitrogen fertilizing and artificial irrigation, either directly or by damming up water; experiments and practical experience have shown that fertilizing as well as irrigation will help to increase pasture supply during this critical period. However, if the same measures are taken during any other part of the season they will increase grass production even more; this applies especially to spring and early summer, but also to late summer and autumn. The curve of the increase in yield obtained with the aid of nitrogen fertilizing or artificial irrigation will thus rise and fall in a line more or less parallel to the curve of grass

regrowth after successive grazings without such fertilization or irrigation. The nitrogen effect is dealt with in a recent publication by E. G. Mulder [4]. Nitrogen fertilizing rather appears to accentuate than to reduce the variation described.

As to artificial irrigation, attention should be paid not only to its effect of supplying the plants with water but also to its cooling the soil and excluding air which will influence the development of the grass plants, i.e. the rate of their passing from one phase to another. Irrigation experts recommend that irrigation should start very early in spring long before the plants begin to run short of water. Having started irrigation at the beginning of plant growth it is continued throughout the season, making the intervals between irrigations as short as possible. This method is said to be more advantageous than what would seem to be a more obvious procedure, i.e. irrigating only when shortage of water supply seems imminent.

These practical results are confirmed by the experiments mentioned earlier. Experimental years in which temperatures are low in spring and early summer and in which precipitation is abundant or the ground water level is kept high always show a much less pronounced midsummer depression than other years. This weather type is quite common in northern Sweden where the soil sometimes remains frozen until the beginning of June or later, and the snow does not disappear completely before the end of May. It is common in that region for the grass to start growing immediately after the snow has thawed away when the soil is still very cold and wet. At this time the deeper soil layers are still frozen — and the soil does not get warmed up or aerated to any considerable degree before the middle or end of June.

Examples of such growth in northern Central Sweden and in North Sweden will be found in the harvesting series (plot groups) of Gisselås for 1939, 1940 and 1941 (cf. Fig. 4*a* and the corresponding weather diagrams in Fig. 4*b*), and in those of Brännberg for 1944 and 1945 (cf. Fig. 5*a* and the corresponding weather diagrams in Fig. 5*b*). In all those cases growth only started towards the end of May, and temperatures were low even in June. There are other years when growth had already started at the beginning of May and when June temperatures were relatively high, viz., Gisselås 1937 and 1938 and Brännberg 1943. In these latter cases the growth diagrams show midsummer depressions, whereas in the former cases, i.e. when

comparatively cold weather prevailed in spring and early summer, midsummer depressions occurred only exceptionally. As stated above, the total experimental material from the Northern parts of Sweden comprises 56 plot groups among which 44 do not show any midsummer depression; cf. my earlier publication [10]) on this material. It may be mentioned that the curves shown in these publication make it seem likely that midsummer depressions actually occurred in four further plot groups. Thus, in the material from North Sweden 71% of the plot groups do not reveal any noticeable depresssion around midsummer.

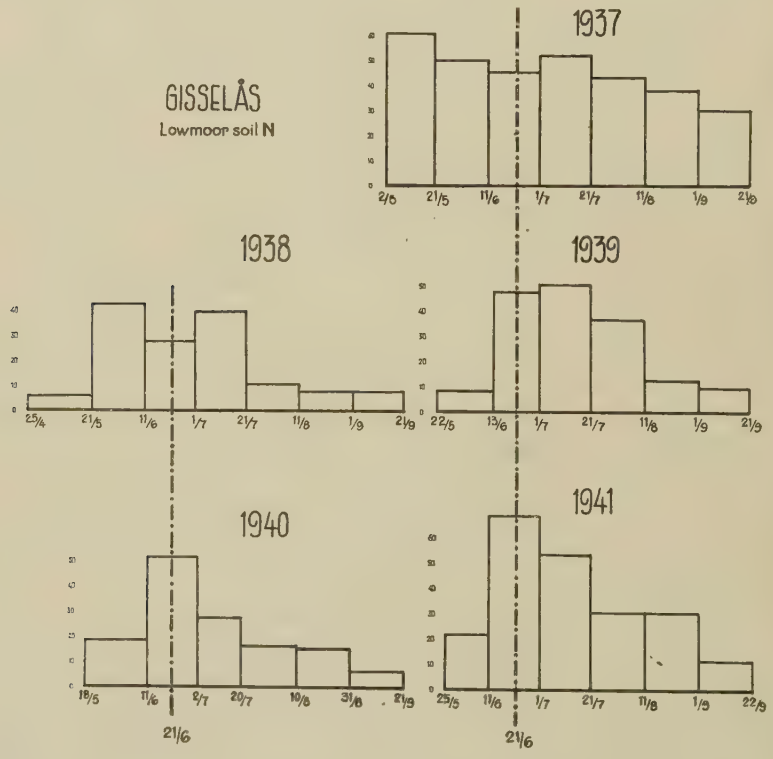


Fig. 4*a*. Simple cutting series during five successive years at *Gisselås* in the interior of northern Central Sweden, 325 m above the sea level; nitrogen fertilized pasture on well drained lowmoor soil. Phosphate and potassium status rather good. Cf. the text under Fig. 1 and the text on page 311.

There have been some experimental years in which similar conditions prevailed in the interior of southern middle Sweden and in

South Sweden too, when due to abundant precipitation from April to June, unusually low temperatures and a low percentage of sunshine hours, soil temperatures were low for a long time while soil moisture was at a maximum. This was the case especially in organogenous soils with a high groundwater level.

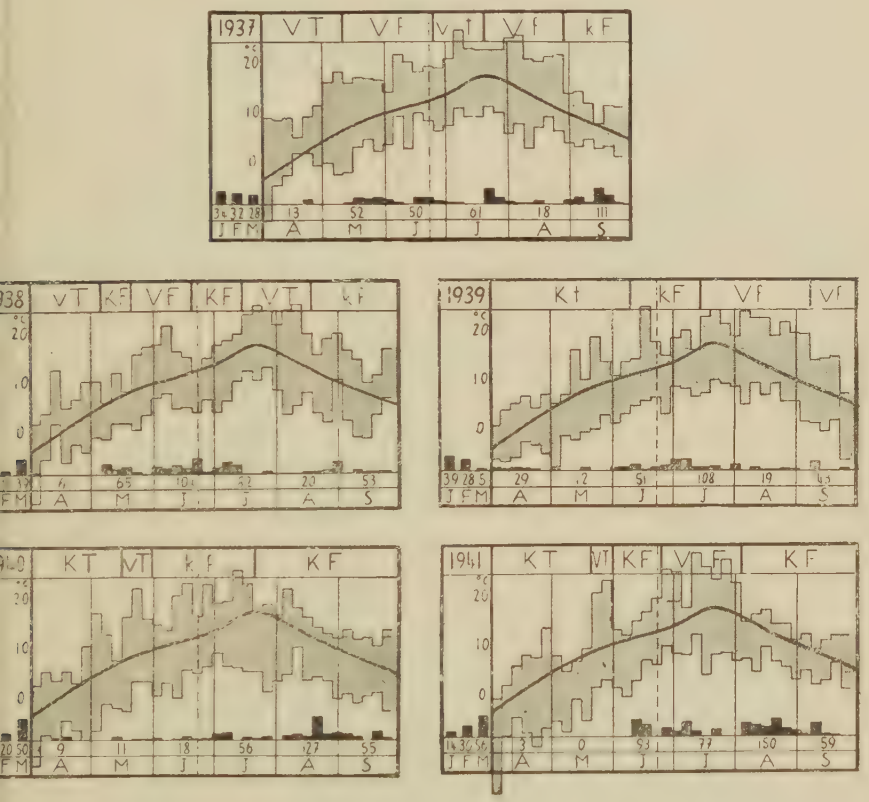


Fig. 4*b*. Weather diagrams for the five experimental years at Gisselâs (Fig. 4*a*). Cf. Fig. 2*b*.

In my material, obtained from more southern regions of Sweden, the two plot groups of Åkerlänna 1944 (Fig. 3*a*) best illustrate this northern growth type. Growth started comparatively late in spring but was vigorous from the very beginning. In this experimental locality the groundwater level was high during years of abundant precipitation since the lowmoor soil on which the experi-

ment was run was poorly drained. Fig. 3*b* shows that nights with temperatures below 0°C occurred up to the very time when growth was assumed to have started. Cold and moist weather prevailed until the end of June. In the growth diagrams for both plot groups no trace of a midsummer depression is visible. A late depression occurs in the diagram of one plot group, but this was probably caused by the high temperature and by the absence of precipitation during the period when in parallel soil experiments *) water consumption was at its highest; for this reason this depresssion should in no way be associated with the midsummer depression phenomenon.

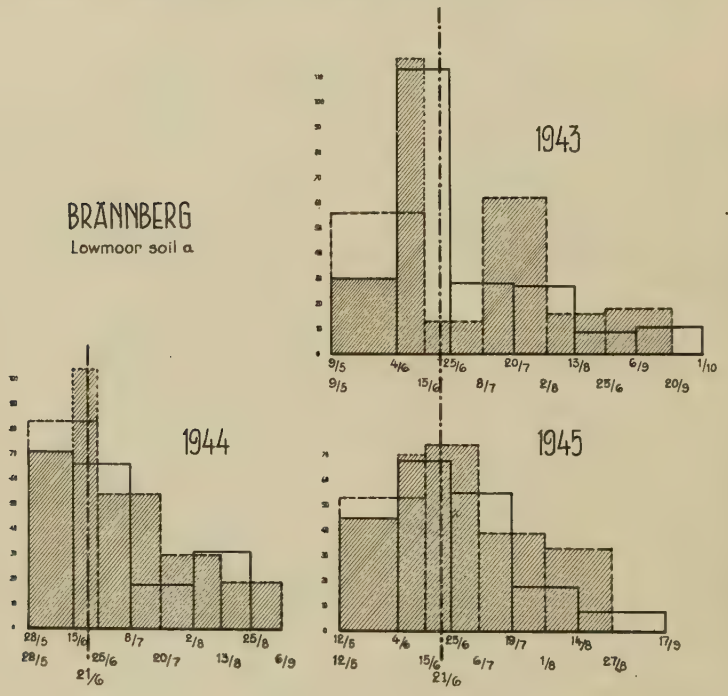


Fig. 5*a*. Double cutting series during three successive years at *Brännberg* not far from the coast of North Sweden, about 80 m above the sea level; pasture not fertilized with nitrogen on well drained lowmoor soil. Phosphate and potassium status good. Cf. the text under Fig. 1 and the text on page 311.

Experimental results show that there generally occurs a comparatively short period of cold and often also moist weather at the time of the midsummer depression; compare Figs. 2*b*, 3*b*, 4*b* and 5*b*.

*) Not yet published.

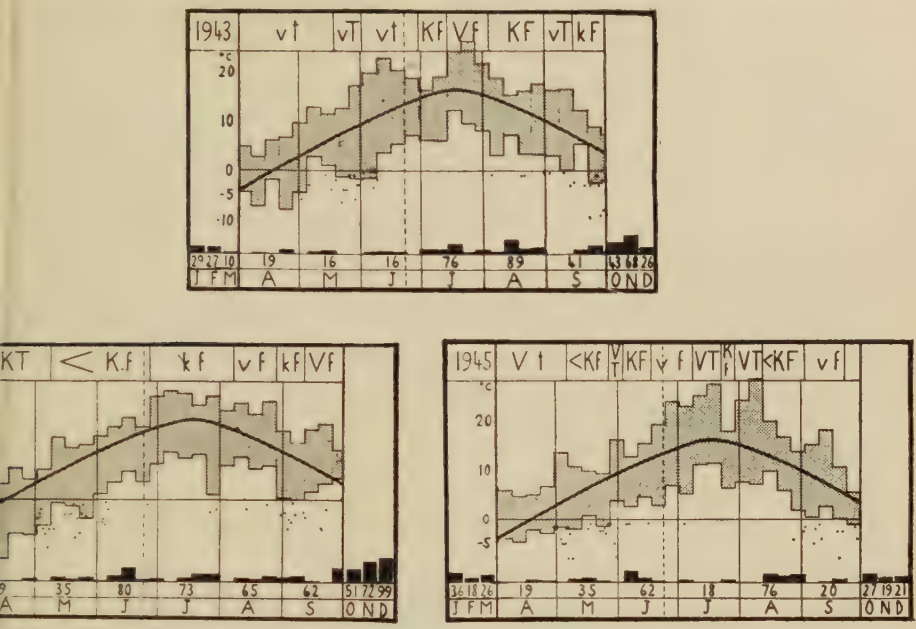


Fig. 5*b*. Weather diagrams for the three experimental year at Brännberg (Fig. 5*a*). Cf. Fig. 3*b*.

Frequently there is a sudden transition from rather warm and dry weather to colder and less dry weather. This weather change may be supposed to act as an immediate cause for the full "onset" of the reproduction phase. On the other hand, whenever comparatively cold and moist weather conditions prevailed during the whole of spring and early summer, and the soil was fairly cold and moist all the time, there is no such definite change of weather; as may be seen from the weather diagrams for the northern type of harvesting series. The grass plants may then be more reluctant to enter upon their reproduction phase. It seems reasonable to assume that this would delay the full start of the reproduction phase without necessarily delaying the time of its end, also. Experiments rather seem to indicate that a "contracted" midsummer depression will occur under the described weather conditions. Full development of the midsummer depresssion seems thus to take place only when (1) spring and early summer have been warm enough and the soil has once been dry enough to allow sufficient penetration of air to the grass

roots, and (II) there is a transition from this weather type to colder and moister weather.

The most striking example of growth without midsummer depression in South Sweden was found in Flahult, 1936, see Figs. 2*a* and 2*b* (1936); the latter shows that cold and moist weather prevailed during late spring and early summer up to the middle of June. Such was also the case for Åkerlänna in 1944 and for most years at Gisselås and Brännberg. The weather at Flahult in 1936 did not include a cold period in the middle of the summer; on the contrary, the weather from the middle of June to the beginning of July, i.e., during the time when midsummer depresssions will appear, was exceptionally warm. As a result, yields of northern type were obtained that ye ar from all the plot groups on Sphagnum soil as well as on lowmoor soil and mineral soil. In no other experimental year were the total yields obtained at Flahult so high as in 1936.

If sunshine conditions could be disregarded completely it would

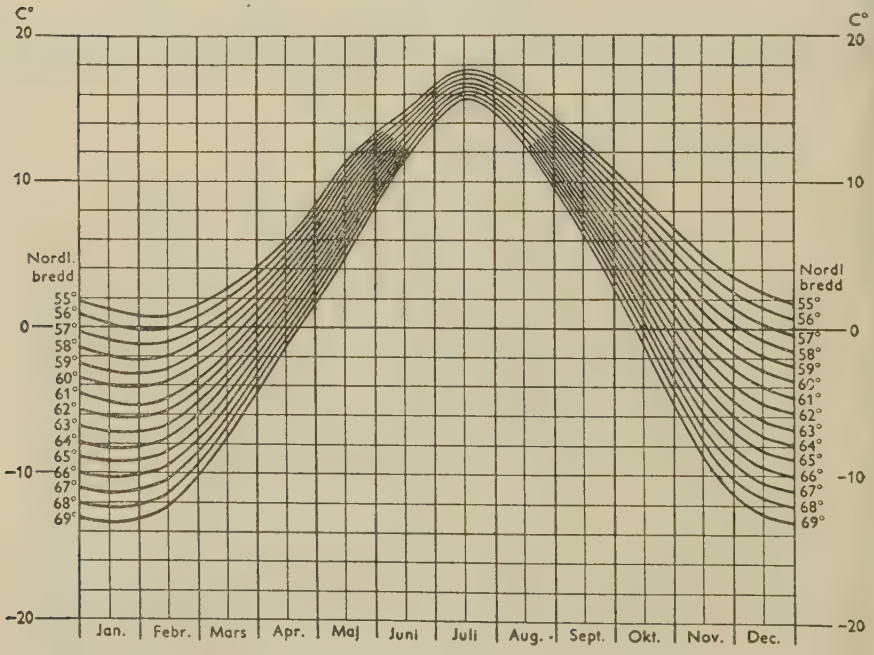


Fig. 6*a*. According to A. Ångström[15]). The variations in "normal temperature" at places of different latitudes in Sweden, reduced to sea level conditions. Note the depression in June, especially marked in Southern Sweden.

seem quite possible that artificial irrigation or damming up of water started early in spring would affect the grass growth in a similar way to the weather conditions described above so as to reduce considerably undesirable variations in pasture supply. The experiments of 1936 seem to indicate that such arrangements would give strikingly positive results during years with hot weather in the midsummer month.

In my earlier publication [10]) concerning these experiments a more or less northern type of distribution of regrowth after grazing occurred, in South Sweden, in 52 out of 132 plot groups according to the rectangle diagrams, and in 37 out of 132 plot groups according to the curve diagrams, that is in 28% of all possible cases. The corresponding data obtained from southern Central Sweden are 49 and 30 respectively out of 80 plot groups or at least 37% of all

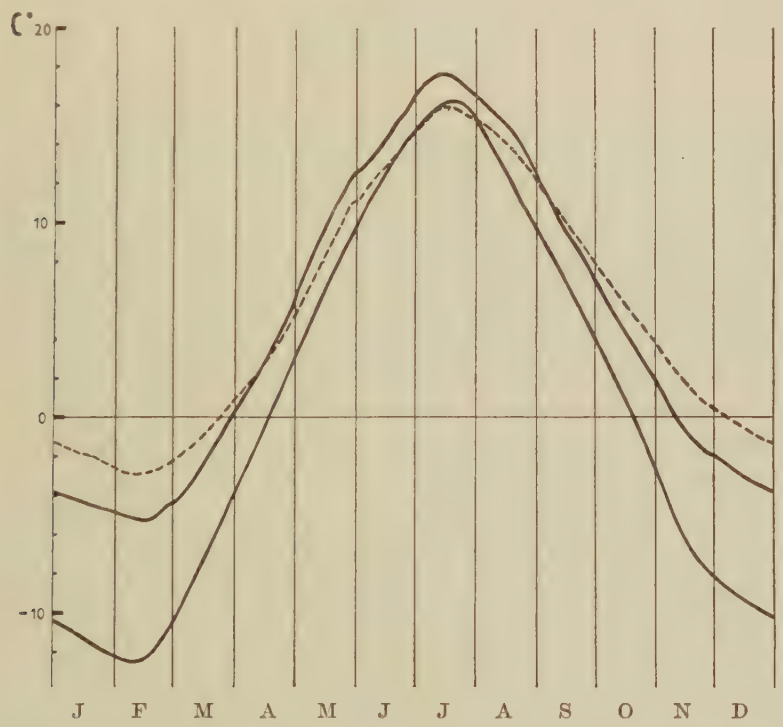


Fig. 6*b*. Annual variation of temperature at 8 a.m. at certain observation stations not far from the experimental localities. Interrupted curve = Flahult region, upper uninterrupted curve = Åkerlänna region, lower uninterrupted curve = Brännberg region.

possible cases. In the Northern parts of Sweden the northern growth type occurs in at least 71% of all possible cases, see above.

In the climate of South Sweden especially a retardation in the temperature increase commonly occurs during June as compared to the temperature increase before and after. This phenomenon, well known to South Swedish farmers, is illustrated in Figs. 6*a* and 6*b*. Fig. 6*a* has been taken from a work by A. Ångström [15]) while Fig. 6*b* is taken from my earlier publication. Both figures show that as one proceeds northwards the retardation becomes less and less pronounced. The relatively cold period that is supposed to bring about the actual onset of the midsummer depression forms a part of the retardation period.

SOME OBSERVATIONS REGARDING THE QUALITY OF THE MATERIAL HARVESTED

As pointed out in my earlier publication [10]) the quality of the material harvested was examined in some of the experiments. Crude protein and phosphoric acid analyses were then made at every harvest. I shall here given the results obtained from a plot group at Flahult (South Sweden) and from one at Gisselås (northern Central Sweden), both with southern growth type, as well as from two plot groups with northern growth type obtained at the same experimental localities during other years; see Table II. The harvests analyzed consisted chiefly of grasses. The clover content varied at Flahult from 1 to 8% and at Gisselås from 0 to 3%, reaching its maximum in June and July. The growth diagrams for these plot groups are given in Figs 2*a* and 4*a*.

The plot groups of southern growth type thus show a certain increase in crude protein and phosphoric acid contents in early summer and around midsummer; this improvement in quality, however, is by no means great enough to compensate for the decrease in quantity occurring at midsummer. On the other hand, in the plot groups of northern growth type the vigorous growth in early summer and around midsummer involves at least in the Flahult series a slight decrease in crude protein and phosphoric acid contents, and this deterioration may of course to some degree reduce the usefulness of the large yields obtained.

TABLE II

Crude Protein and Phosphates; per cent of Dry Matter in Harvesting Series of Southern and Northern Types. (The phosphate and potassium status of the soils subjected to harvesting was rather good or good.)

Cutting Dates	Southern Type				Northern Type			
	Flahult 1937		Gisselås 1937		Flahult 1936		Gisselås 1939	
	Crude Protein	P_2O_5	Crude Protein	P_2O_5	Crude Protein	P_2O_5	Crude Protein	P_2O_5
1/5–2/5	25.3	1.43	—	—	25.6	1.17	—	—
21/5–24/5	20.8	1.20	17.7	1.16	28.3	1.32	—	—
11/6–13/6	*21.1*	*1.27*	*18.1*	*1.43*	—	—	23.3	1.11
20/6	—	—	—	—	*17.7*	*1.14*	—	—
30/6–1/7	*24.2*	*1.48*	*19.3*	*1.27*	—	—	*21.2*	*1.16*
21/7	—	—	16.4	1.21	*18.5*	*1.35*	19.7	1.23
3/8	22.7	1.30	—	—	—	—	—	—
11/8	—	—	18.8	1.18	—	—	21.3	1.25
20/8	—	—	—	—	20.9	1.54	—	—
1/9	23.1	1.67	22.3	1.27	—	—	21.7	1.28
21/9	—	—	22.6	1.10	19.5	1.53	21.3	1.11
30/10	25.6	1.41						

Experiments are still being carried on with the object of investigating seasonal variations in the regrowth of grass plants after successive cuttings. The occurrence and character of the midsummer depression in regions of different climate is to be studied as well as the morphological signs of development of different species of pasture grasses.

REGIONAL INVESTIGATIONS NOW GOING ON; PRELIMINARY RESULTS

A regional investigation of the rhythm of regrowth on good established pastures is being made in cooperation with a great number of experimenters. Care has been taken to choose, as far as possible, such experimental localities where water supply is good throughout the year due to abundant rainfall or a high ground water level. At present experiments are being run according to substantially identical plans in Sweden, Norway, Finland, Denmark, Iceland, Holland and Austria; perhaps it will be possible to have experiments laid out also in countries farther south. The experimental locality situated farthest to the north is Tromsö in northern Norway.

These experiments are being carried out from 1949, essentially in accordance with the following plan: The plots available are divided into six equal groups (treatments in latin squares or other arrangements). In each plot group cuttings are made at intervals of four and two weeks alternately but the cuttings of each group are *inter se* displaced by one week. If, thus, group 1 is harvested for the first time one week after growth is assumed to have started in spring, group 2 will be harvested for the first time one week later, group 3 two weeks later etc., group 4 thus being harvested three weeks later or four weeks after the assumed beginning of growth. This first harvest is called an "A harvest". In group 5 the first harvest is taken 7 days and in group 6, 14 days after the beginning of growth; these harvests are called "B harvests". Futher B harvests are taken 14 days after the A harvests in all the six plot groups, and 28-days after each B harvest another A harvest is taken, and so on. In this way each plot group comprises A harvests (28-day-old plants except in the first A harvest) alternating with B harvests (14-day-old plants) during the whole season. The season is thus divided into harvesting periods of six weeks each comprising one A harvest with 28-day-old plants and one B harvest with 14-day-old plants.

All B harvests follow upon A harvests, and are therefore comparable *inter se*, and similarly all A harvests, with the exception of the first one in spring, follow upon B harvests and are therefore also comparable. The aim of this investigation is only to compare successive harvests of plants of the same age. By means of alternating A harvests with B harvests it is possible to study the relative growth under such short terms as 14 days without harming the production of the sward as much as would be the case if all the harvest intervals for each plot were 14 days only. The exclusive use of 28-day intervals would not show, with sufficient clearness, at what time the main phases in the development of the grass plants would occur, especially the reproduction phase around midsummer. In most parts of Sweden 14-day intervals are quite suitable; in more southern regions 21 days would possibly be a sufficiently short time for B harvests. The season would thus be divided up into intervals of seven or eight weeks. Average daily growth is of course much greater in northern countries.

The following diagram will explain this arrangement still more clearly:

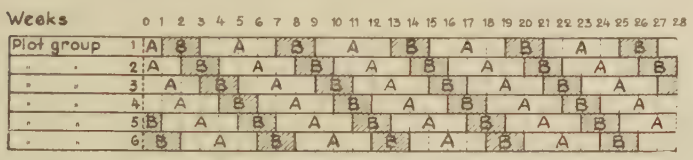


Fig. 7*a*. Distribution of A and B harvests in the experiments running since 1949. Numbers in horizontal top line = numbers of weeks from assumed start of growth; numbers in vertical line to the left = numbers of plot groups.

The aim of this system was to obtain, from all the plot groups together, four continuous series of A harvests (28-day intervals) and two continuous series of B harvests (14-day intervals). In places north of the polar circle and in Iceland A harvests were taken after 20 days and B harvests after 10 days of growth only.

We thus obtain the following four continuous series of A harvests;

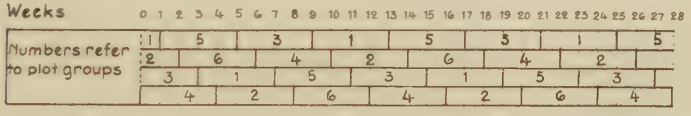


Fig. 7*b*. Four week series obtained according to the plan shown in fig. 7*a*.

and the following two continuous series of B harvests.

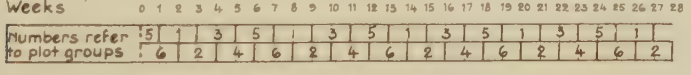


Fig. 7*c*. Two week series obtained according to the plan shown in fig. 7*a*.

In the two first years of the regional field experiments (1947–1948) only A harvests were taken, according to the following plan:

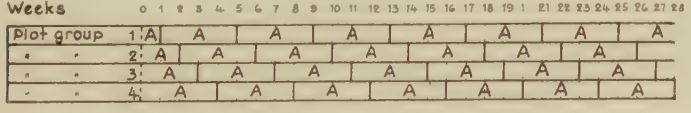


Fig. 8. Arrangement of harvests in the experiments of 1947 and 1948.

Experimental results of 1947–48 obtained according to this latter harvest system will only reveal depressions of rather long duration, but with the shorter harvest intervals applied from 1949 onwards it will be possible to make a more thorough investigation into seasonal variations in the rate of pasture regrowth after grazings.

The following table will give a rough survey of the preliminary results obtained in the experiments of 1947 and 1948; it shows how curves of the southern type (two top curves) and curves of the northern type (one top curve) are distributed over places of different regions.

TABLE III

Numbers of diagrams from experiments carried out during the years 1947–1948 each with four groups of plots the cutting dates of which are *inter se* displaced by seven or five days

Region	Geographical location and latitude	Diagrams with "true" depressions in the middle of the summer	Diagrams with depressions merely due to cold weather	Slight traces of depressions	Diagrams without depressions in the middle of the summer	Total
I	Netherlands and South Austria 47°–54° N.lat.	6	—	1	—	7
II	South Sweden 55°–58° N.lat.	7	—	1	3	11
III	Southern Central Sweden, South Norway and South Finland; 59°–62° N.lat.	4	—	1	4	9
IV	Northern Central Sweden and North Sweden; 63°–65° N. lat.	1	—	2	3	6
V	Sweden and Norway north of the Pole Circle; 67°–70° N. lat. and Iceland 64°–66° N.lat.	1	4	—	2	7
					Total	38

No further statements can be made at this preliminary stage, but more details will be published later on.

The results of the experiments carried out in 1933–1945 are all published in Swedish with English Summaries [7] [8] [9] [10] *).

*) These publications can be ordered from the author directly. Address: Vassmolösa, Sweden.

In working out the experimental data of 1933–45 it was observed that midsummer depressions appear more and more clearly as one proceeds further south. The preliminary data in Table III obtained from the first two years seem to give further evidence for this fact.

Moreover the present experiments from 1949 onwards, which due to the new plan as outlined above permit a closer study of the various phenomena, seem to prove the correctness of my earlier assumption that midsummer depresssion occurs also in places far up in the north, although during a sharply reduced period of time. The experiments further appear to confirm what to some extent could be gathered from the old experimental material already, viz., that average daily production tends to show a period of small increase near the end of the vegetation period.

EXPERIMENTS WITH INDIVIDUAL GRASS SPECIES; PRELIMINARY RESULTS

The seasonal rhythm of individual species of pasture grasses is being studied at the auhor's experiment station at Christinelund (postal adress Vassmolösa). Cuttings are performed according exactly to the same plan as that applied to the regional experiments from 1949 onwards. The grasses are grown in concrete vessels ($30 \times 30 \times 30$ cm) asphalted on the inside. In the bottom of each vessel there is a hole with a water inlet tube. A little earthenware bowl is turned upside down on top of the tube opening, and above this bowl lies a 5 cm gravel layer (5–10 mm diam.) spread over the whole area of the bottom of the vessel. On top of this there is a further layer of coarse sand up to 10 cm above the vessel's bottom. The rest of the vessel is filled with a mixture of 50% of good garden soil and 50% of sand (1–2 mm particles). Each of the 224 vessels has been sown with one of the ten grass species most common in Sweden, mainly high-bred varieties. Each of the three species which are of the greatest interest for south-Swedish farmers (*Lolium perenne*, *Poa pratensis* and *Dactylis glomerata*) have also been sown in 20 larger circular concrete vessels (60 cm diam., 120 cm depth). These vessels, with gravel, sand and soil arranged in a way similar to the above, are in addition provided with an arrangement for varying the height of the ground water level.

Irrigation of the vessels is carried out with the aid of a hydro-

phore, pumping water from a near-by river into the vessels from below. As soon as the water surface has risen above the soil surface the water is tapped off, air being led in through special valves. Soil moisture is controlled every day, and irrigation is then applied in accordance with the soils' requirements, every or every other day, or every three or four days, depending on weather conditions. It is hoped that this "pulsating irrigation" will bring about an optimum water supply without excluding the air. Nutrients are supplied to the vessels in aqueous solution once a month, together with irrigation.

The vessel experiments have now been run for one year (the grasses were sown in August 1949), and so far growth has been satisfactory.

In accordance with the plan explained above there is every week one A harvest and one B harvest on some vessels of each species. The grass material harvested is immediately air-dried. Before each harvest some typical plants are selected for a herbarium. My object is to study those morphological changes in the plants during the season which may give an indication of the main developmental phases that have been supposed to be associated with the changes occurring in daily growth.

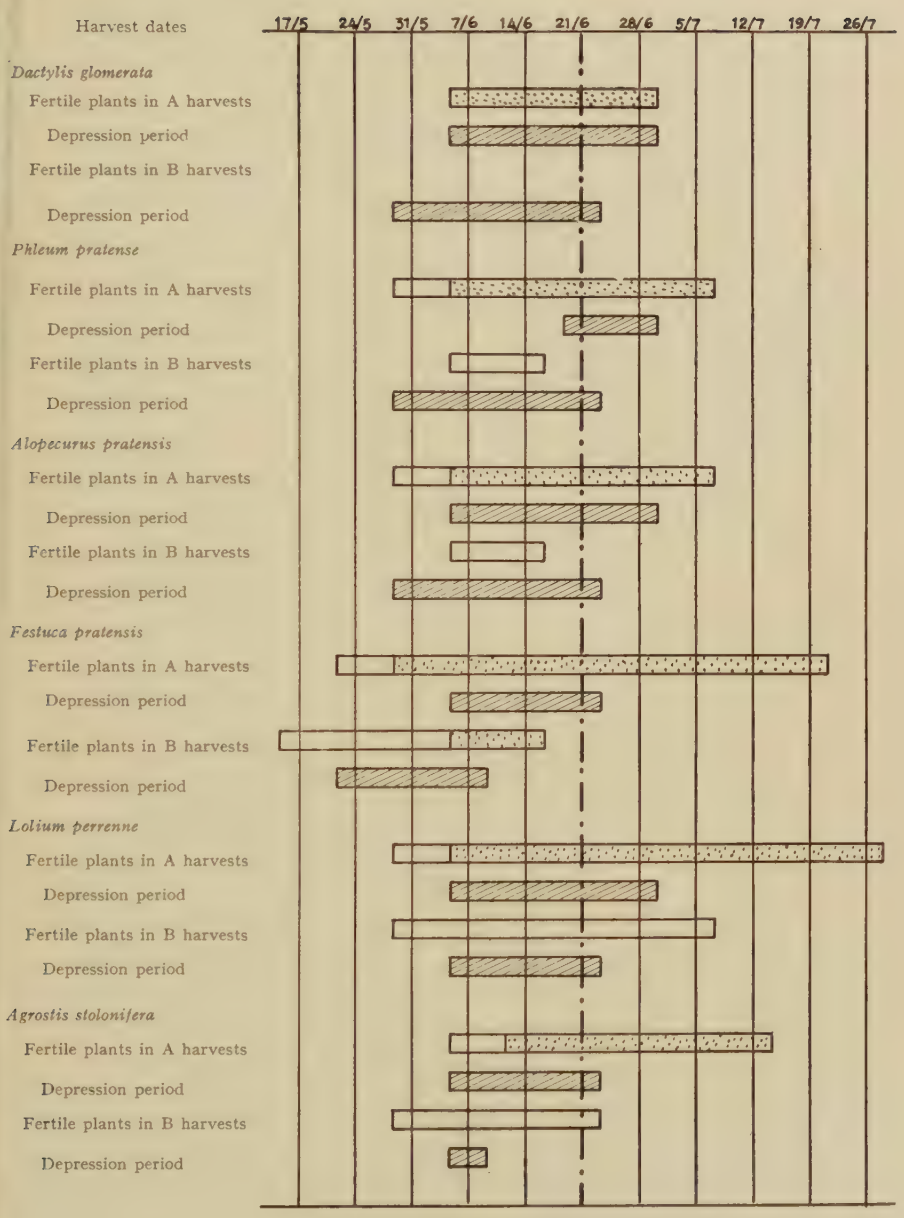
Fig. 9 gives a rough survey of the results obtained in 1950 for the six species that have already in the first year produced fertile plants in B harvests as well as in A harvests. Spike formation occurred in A harvests and in one case even in a B harvest.

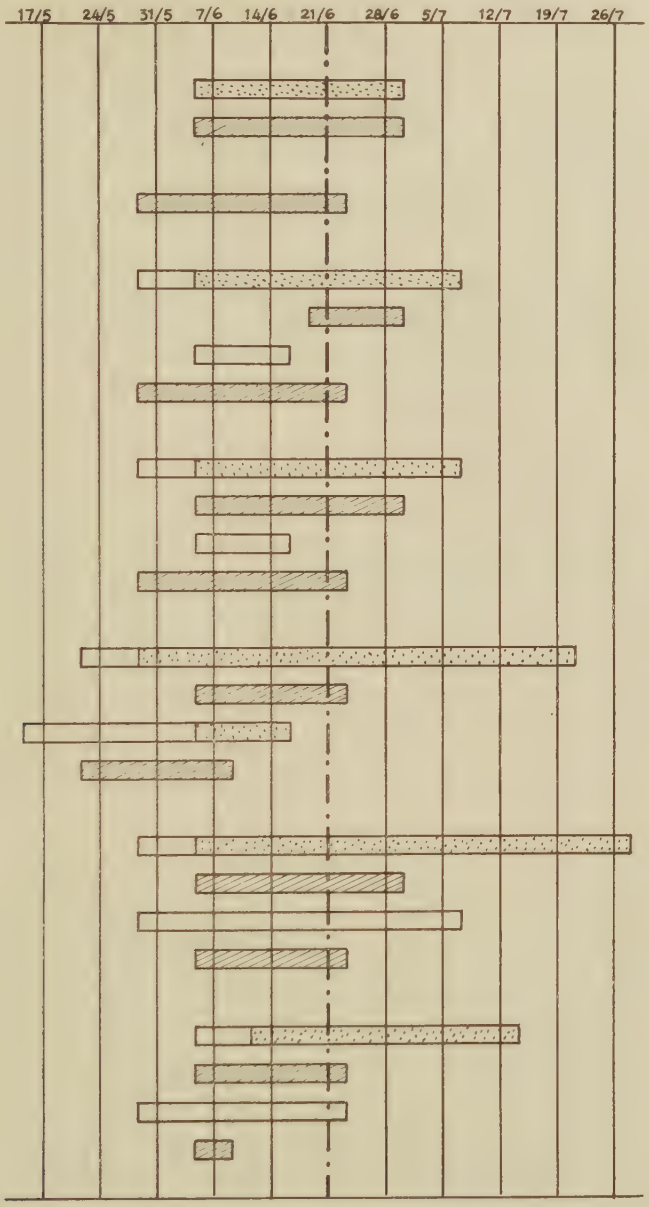
In the table, harvesting dates during May–July are indicated by vertical lines. Striped rectangles mark out the duration of depression periods (cf. the figure with text) while white and dotted rectangles mark out the periods during which fertile plants were found, the dotted parts indicating periods during which also some plants with fully developed spikes were found.

A look at the table will show that there is quite a striking coincidence of the periods during which fertile plants were obtained and those during which plant regrowth after cuttings was markedly depressed. Inflorescence begins at about the same time in all species but terminates much later in *Lolium* and *Festuca* than in the other species. The last part of the *Lolium* and *Festuca* inflorescence periods, however, showed much weaker flowering activity than the earlier parts of the periods. Depression often occurred earlier in B harvests

ERRATUM

(fig. 9, p. 335, vol. III).





"Depression periods" = periods extending from the first harvest data at which yields show a drop as compared to the preceding harvests to the first harvest date after which yields again show a rise as compared to the preceding harvests.

Periods in which harvests contain fertile plants with spikes perceptible by the eye.

Periods in which harvests contain fertile plants without spikes perceptible by the eye.

Fig. 9. Periods of depression in yield and periods during which the harvest material contained fertile plants; A harvests (28-day-old plants) as well as B harvests (14-day-old plants); six different grass species.

than in A harvests; in the latter the depression was generally around midsummer.

The more or less simultaneous occurrence of reduced production and of the presence of fertile tillers seems to confirm my working hypothesis that there occurs a reproduction phase in the development of our pasture grasses which may be one principal cause of the reduction in average daily growth observed in midsummer. The grasses studied appear to have such a strong tendency towards reproduction at a certain time of the season that they adjust their whole life-cycle to this purpose even when exposed to the injury of cuttings every 28 and 14 day respectively.

SUMMARY

In practical farm management, it was observed that a change in the grazing schedule of a group of pastures seemed to cause a change in their individual yields. This phenomenon, the "relative change in yield" was confirmed by experiments carried over three years [9] [10]. These experiments have shown that the relative change in yield does not only comprise the total annual production but also its distribution over the grazing season. A first cutting carried out at a certain time will increase spring and early summer production at the cost of late summer and autumn production while a first cutting at another date will have the opposite effect. Fig. 1, Fig. 3*a* and Fig. 5*a* are illustrations of this phenomenon. Certain practical consequences are discussed.

Another observation made was the "midsummer depression", i.e. pasture regrowth seemed much weaker after grazings at the end of June and beginning of July than at other times, and this appeared to be independent of rainfall conditions. Eleven years of experiments [7] [8] [10] have proved the actual occurrence of this phenomenon but have likewise shown it to apply to the grass only. Midsummer depression appears more clearly in southern regions than in regions of more northern latitudes. Figs. 2–5 as well as Table I will illustrate this observation. Table II gives some figures from parallel studies of quality.

My working hypothesis for explaining the phenomena mentioned above is that the grasses are assumed to pass through a series of developmental phases to which they are bound by genetic constitution. Probably in some phases grasses are more sensitive to the injury of a cutting than in others which may explain their different reactions when cut for the first time at different dates in spring ("relatieve change in yield"), and it may also explain the midsummer depresssion phenomenon: around midsummer the grasses are assumed to pass through their reproduction phase which covers conversions of substances already assimilated rather than increase in weight and which corresponds to the time of flowering and seeding. It is quite

remarkable that this phase seems to occur even if cuttings prevent the plants from ever reaching the flowering stage.

This fact is further evidenced by experiments now going on with single grass species cut at certain intervals. Morphological examinations of *Dactylis glomerata, Phleum pratense, Alopecurus pratensis, Festuca pratensis, Lolium perenne* and *Agrostis stolonifera* have shown a great number of plants to be definitely fertile from about the beginning-middle of June to some time in July. However, these experiments have so far been run for one season only (see Fig. 9).

A short description is given of the methods used in a regional investigation now going on. The aim of this is to study the rhythm of regrowth in ordinary pasture swards. This investigation has been carried on since 1947 in co-operation with heads of experiment stations in all the Scandinavian countries, as well as in Iceland, The Netherlands and Austria (compare Figs. 7 and 8). From the two first experimental years 1947–1948 some preliminary results are given in Table III which further support my earlier observation that midsummer depresssion occurs more frequently in the South than in the North.

Some practical measures for counterblancing the damage done by the midsummer depression are discussed in the essay, in particular artificial irrigation and nitrogen fertilization.

Finally it is pointed out that many of the results discussed here must still be regarded as preliminary.

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LITERATURE

1) Frankena, A. J., Enkele beschouwingen voor theoretische graslandvragen. Landbouwkundig Tijdschrift **52** (1940).
2) Gjöbel, G., Experiments on the Use of Nitrogen on Swedish Pastures. Rep. Fourth Internat. Grassland Congress (1937).
3) 't Hart, M. L., De seizoenschommelingen van de grasgroei in Nederland. Maandbl. Landbouwvoorlichtingsd. **5**, 137 (1948).
4) Mulder, E. G., Onderzoekingen over de stikstofvoeding van landbouwgewassen. Landbouwkundig Bureau der Nederlandse Stikstofmeststoffenindustrie 's-Gravenhage (1949).
5) Nilsson-Leissner, G., The influence of certain metorological factors upon the growth of white clover-and some pasture grasses. Botaniska Notiser, Lund (1935).
6) Nilsson-Leissner, G., Olika arters och stammars inverkan på betenas säsongvariation. Sv. Vall- o. Mossk.för. kvart. skr. **6**, 24 (1944).
7) Rappe, G., Variationer i betestillgången under säsongens olika delar. (With an English Summery). Sv. Vall- o. Mossk. för. kvart. skr. **6**, 5 (1944) .
8) Rappe, G., Några iakttagelser rörande valltillväxt och vattentillgång. (With an English Summery). Ibid **8**, 1 (1946).
9) Rappe, G., Utjämning av betestillgången genom ändamålsenlig användning av olika jordslag. (With an English Summery). Ibid **9**, 3 (1947).

10) R a p p e, G., Några för betestillväxten karakteristiska grunddrag. (With an English Summery). Sv. Vall- o. Mossk. för. Meddelanden nr. **14**, 581 (1948).
11) R o m e l l, L. G., Något om rythm och periodism hos växter. Med. Fören. Tidskr. nr **1**.
12) S t a p l e d o n, R. G., The Seasonal Productivity of Herbage Grasses with particular reference to different systems of cutting etc. Bull. Welsh Plant Breeding Sta., Series H, nr. **3**, 15 (1924).
13) W h y t e, R. O., Crop Production and Environment. London (1946).
14) W o o d m a n, A. E., B l u n t, M. A. and S t e w a r t, W. A., Nutritive Value of Pasture. II Seasonal Variations in the productivity, botanical and chemical composition, and nutritive value of Pasturage etc. Journ. of Agr. SC. XVII, II (1927).
15) Å n g s t r ö m, A., Lufttemperatur och temperaturanomalier i Sverige 1901–1930. Medd. fr. Statens Meteorol. Hydrogr. Anst. Bd. 7, 2 (1938).

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METABOLISM OF PLANTS AFFECTED WITH LIME-INDUCED CHLOROSIS (CALCIOSE)

II. ORGANIC ACIDS AND CARBOHYDRATES

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In a previous rapport [6]) which dealt with the nitrogen metabolism of chlorotic plants, it was established that lime-induced chlorosis is characterized by unbalance between synthesis and analysis of proteins. Such unbalance is reflected in the modifications that occur in certain of the nitrogenous components, especially in total nitrogen, soluble nitrogen, protein nitrogen, and residual nitrogen. The present account, which is an integral part of the same investigation, deals with correlated modifications, as reflected in differences between the foliage of normal and chlorotic plants in content of certain organic acids and carbohydrates.

MATERIALS AND METHODS

Seventeen species of plants, grown in fields and gardens near Vienna, Austria, as previously indicated, were sampled in these studies that extended over the period 1941 to 1944. Potted lupines were employed as material for part of the analyses. In some instances series of samplings of leaves were made systematically each year throughout the entire growing season and in others only occasional specimens were taken to secure indications of the course or trend of changes.

The analyses sought to measure quantitatively such alterations as those in citric acid, malic acid, tartaric acid, and total organic acids, and also starch, sugar, and total carbohydrates. In general, use was made of expressed sap, and the results are stated in terms of weight of constituent in milligrams per one gram of dry weight of material.

To determine citric acid an alcoholic solution of barium chloride (3 : 1) was used as a precipitant. After centrifugation in 15 ml tubes the precipitate was washed with 70% alcohol and then dissolved in weak acetic acid orweak hydrochloric acid and brought to a volume of 2.5 ml with water. After centrifugation two 1 ml samples of liquid were taken for parallel determinations. To each sample was added several drops of 20% potassium bromide and several drops of dilute sulphuric acid (1 : 4). The tubes containing the samples were next placed in a water bath at 43°C after which sufficient saturated potassium permanganate was added to decolorize completely. Any excess of permanganate was removed with ferrosulphate or oxalate. The material was allowed to remain in the bath until the next day to permit the formation of pentrabrom acetate. After centrifugation, kaolin was added to collect precipitate adhering to the tube walls, and again the tubes of material were centrifuged. The supernate was then poured off the tightly compacted precipitate. Water was added and mixed with the precipitate by stirring with a glass rod. After being again centrifuged, two or three more washings with water and centrifugations were made. The thoroughly washed precipitate was next mixed with 2 to 4 ml of alcohol, poured into a beaker, heated to the boiling point of alcohol, and 0.5 to 2.0 ml of 20% alcoholic solution of sodium iodide were added. This solution was titrated against a solution of sodium thiosulphate in the presence of starch, 1 ml of 0.02 N sodium thiosulphate being regarded as equivalent to 0.7 mg of citric acid.

It was not possible to secure pyridine, the reagent which is usually employed in determination of malic acid, because of conditions incident to World War II. Hence a less direct method, as follows, was required. To the expressed sap was added barium hydroxide in alcoholic solution (1 : 3). The precipitate that formed was washed with 70% alcohol, then dissolved in acetic acid, neutralized, and alcohol added to make 28% alcohol. From the solution thus formed citric, succinic, and tartaric acids are precipitated and barium malate remains in solution. The precipitate of these acids was separated by centrifugation, the supernate was removed, and to it alcohol added to make 80% alcohol, thus precipitating the barium malate. Nitric acid was added in sufficient amount to liberate the barium and from the amount of nitric acid required the quantity of malate present was calculated

Tartaric acid was precipitated from the sap as potassium bitartarate by use of potassium chloride, acetic acid, and alcohol. The precipitate obtained by centrifugation was washed with 50% alcohol to remove the acetic acid, dried, and finally titrated, using 0.02 N sodium hydroxide, with phenolphthalein as an indicator.

Those acids precipitable in alkaline solution of barium hydroxide are regarded as "total organic acids". The solution of barium hydroxide was added to expressed sap in the presence of phenolphthalein until there was a slight excess of alkalinity, and to the mixture 3 volumes of alcohol were added. The precipitate which formed was centrifuged, washed with 70% alcohol, after which the barium was freed by use of concentrated nitric

acid. Barium was then determined quantitatively, and on this basis total organic acids were calculated.

Sap was used for total carbohydrate determinations after dilution with water, clearing with lead acetate, and inverting with dilute sulphuric acid, the materials being maintained at 80°C.

Dry material was used to measure the quantity of starch. The leaf tissues were extracted with a concentrated calcium chloride solution, then the precipitate which formed, after addition of iodine, was carbonized by use of a mixture of sulphuric acid and potassium bichromate. By this means the amount of starch in the sample could be computed.

RESULTS

Citric Acid.

Preliminary analysis of apparently normal leaves of various species in comparison with those that were manifestly chlorotic in different degrees of severity indicated that chlorosis is correlated with increased elaboration of citric acids. Data for these preliminary analyses are assembled in Table I.

TABLE I

Content of citric acid, as mg/1 g dry weight, in certain species of healthy and chlorotic plants

Species	Green	Slightly chlorotic	Moderately chlorotic	Strongly chlorotic
Cydonia oblonga . . .	3.8	9.2	17.4	32.5
Pyrus communis . . .	5.6–6.2	7.4	25.2	25.7
Ailanthus glandulosa .	4.4–5.5	11.8	—	16.7
Cornus mas	1.2	—	—	15.0
Cornus sanguinea . . .	0.8	4.5	—	10.9
Viola odorata	6.5	—	18.8	—
Robinia pseudacacia . .	—	11.2	23.4	27.3
Philadelphus coronarius	—	4.4	7.9	—

Manifestly a significant increase in production of citric acid occurs in chlorotic plants, and it is present in largest amount in foliage that is most markedly chlorotic.

Since it was early found in *Fraxinus excelsior* leaves that the titrable acidity approximates 0.01 N whereas that of chlorotic leaves may be 0.36 N, series of analyses were made throughout the growing season to learn something of the course of changes involving production of citric acid, as shown by results in Table II.

In general, from late spring until early autumn there occurs a gradual increase in content of citric acid. This increase is relatively

small in amount in healthy trees as typified by tree No. 1. The leaves of diseased trees Nos. 2 and 3, had approximately five to twenty times as much citric acid as did tree No. 1. It should be stated, however, as regards trees Nos. 4 and 5, that the leaves were to all appearances healthy in spring but by July they were plainly yellowed and by late August and early September the green color had quite completely disappeared. These trees therefore were determined, from biochemical analyses, to be diseased prior to exhibition of visible symptoms.

TABLE II

Progressive increase throughout summer in citric acid content of *Fraxinus excelsior* leaves

No. of tree	Condition of leaves	Successive intervals of approximately a month and mg/1 g of dry weight			
1	Normal	0.5	1.8	2.2	3.0–4.0
2	Slightly yellowed	6.9	11.8	14.4	19.6
3	Very markedly yellowed . . .	28.8	41.2	59.3	82.0
4	Apparently normal	0.5	12.3	14.4	59.3
5	Apparently normal	1.3	10.5	30.7	46.3

A similar trend of change in content of citric acid, occurred in the foliage of *Sambucus nigra*. In one healthy bush, during the period from spring to autumn, the increase ranged from 0.4 mg to 2.3 mg per g of dry weight, in another from 0.2 mg to 2.8 mg; and in another from 0.7 mg to 2.6 mg. On the other hand the citric acid content of a chlorotic bush was 30.0 mg per g of dry weight in spring and by early autumn had increased to 156.0 mg. It is of interest to note that by late autumn the citric acid content had declined to 59 mg and that during the same period the quantity of citric acid in young sprouts on these bushes decreased from 113 mg per g of dry weight to 59 mg. As causes of this decline there are at least two correlated factors, the severity of the disease and the amount of soluble calcium of other bases in the sap. This latter factor will be considered in detail in a subsequent report. It need only be stated at this time that increase in citric acid and in calcium are closely and directly correlated.

Three varieties of apples, Noble, Bellflower, and Reinette, were used in analyses of their content of citric acid, the observations extending over four seasons. Typical results with apple foliage, from a series of tests begun on May 11, 1943 and terminated on Oct. 5 of

that year, and involving a healthy and diseased tree of each variety, are portrayed in Fig. 1. It may be said in explanation from visible evidence of the normal trees that the Noble apple tree was entirely normal whereas the selected trees of the other two varieties were already slightly affected. Moreover among the selected diseased trees, the Noble apple tree was least chlorotic, the Bellflower was intermediate, and the Reinette was severely yellowed.

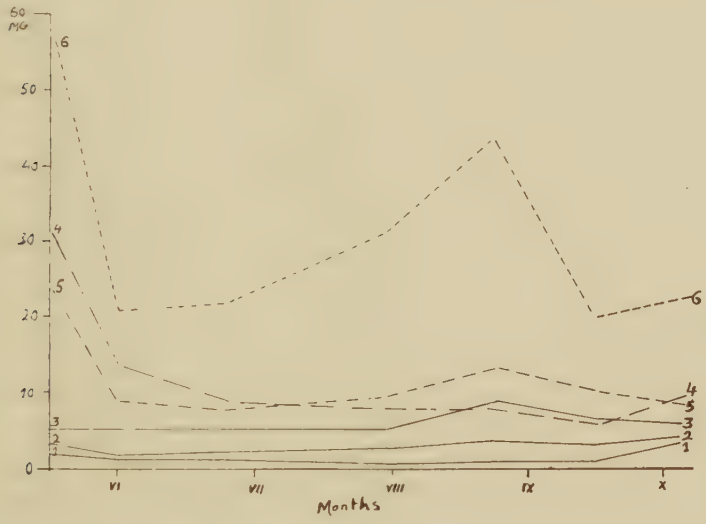

Fig. 1. Content of citric acid in sap of leaves of *Malus domestica*, in mg per 1 g of dry weight. Green plants 1, 2 and 3; chlorotic plants 4, 5 and 6. The varieties: 1 and 4, Noble; 2 and 5, Bellfower; and 3 and 6, Reinette.

When the contents of citric acid of normal trees (Nos. 1, 2, and 3) and diseased ones (Nos. 4, 5, and 6) are compared, the results corroborate the general findings with other plant species, as indicated above. The slight but definite increases during August were probably due to stresses in climatic conditions.

The tests with apple trees made in 1941 and 1942 revealed that trees may exhibit no symptoms of lime-induced chlorosis, as judged by the greenness of the foliage, yet the disturbance in citric acid balance may be very marked. For example, it was found that the content of citric acid of the young leaves of two apparently healthy trees was 0.3 mg and 9.9 mg per g of dry weight, respectively. The older leaves on the former tree remained green and were found

eventually to have a citric acid content of 1 mg per g of dry weight whereas the other tree, by the time external symptoms of chlorosis were first apparent, had a citric acid content of 27.5 mg per g of dry weight.

If there occurs a progressive decrease throughout the summer, in citric acid content of affected trees the trend is toward recovery. Trees exhibiting such recovery were observed in 1944, and recovery is correlated, as will be shown in a subsequent report, with increase in calcium content. Apparently complete recovery may be made by occasional trees but, generally, affected trees eventually perish. In trees that are recovering, if the leaves are already obviously yellowish, there may be no return of the normal green color, but in them the rate of citric acid synthesis may have changed to the extent that its content ranges between 4 to 6 mg per g of dry weight. On the other hand green leaves on near-by trees with incipient chlorosis may have 8 to 10 mg of citric acid per g of dry weight. In the latter trees however, the amounts of citric acid would be found in subsequent tests to increase progressively with time.

The studies, using the foliage of two varieties of cherry, Knorpel and Johannes, involved series of analyses made throughout the growing seasons of 1943 and 1944. The latter variety is known to be the more seriously affected with chlorosis. The results of determinations of content of citric acid for normal and diseased Johannes cherry foliage are shown in Fig. 2.

The higher citric acid content of diseased foliage when compared with normal foliage, as shown in Fig. 2, is typical of lime-induced chlorosis in cherry, and the general trend exhibited by this species verifies the findings with each of the other woody plant species studied.

The course of metabolism of citric acid in three species of lupines, *Lupinus albus*, *L. luteus* and *L. angustifolius*, is of a somewhat different pattern from that in the woody species studied. Citric acid may accumulate in the cotyledons and, if the soils are not well supplied with lime, the young leaves may be chlorotic but later-formed ones may be quite normal. If soil conditions are such that the disease becomes severe, citric acid accumulates up to the time that growth is checked and then a gradual decrease in citric acid follows. In white lupine very little citric acid is produced, and then the larger amounts are always present in the older leaves of affected

plants. Further studies with lupines are needed to clarify the correlation of citric acid metabolism with the course of the disease induced by lime.

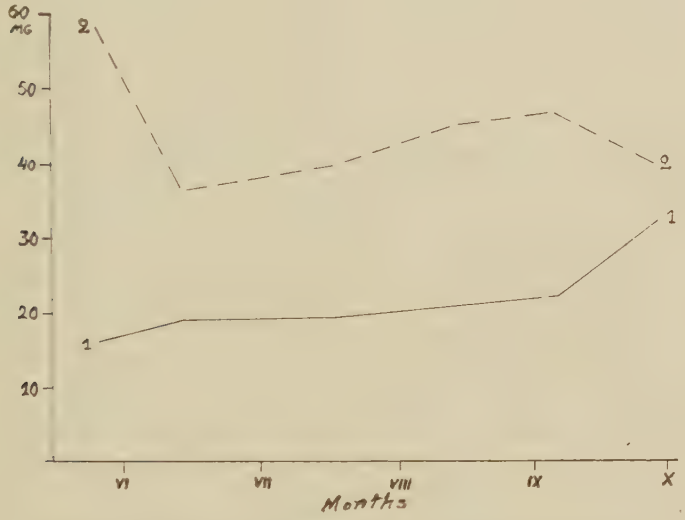

Fig. 2. Content of citric acid in the sap of leaves of normal (1) and chlorotic (2) Johannes cherry.

The two varieties of grape, Rheinriesling and Rotgipfler, that were analyzed, also exhibited certain differences in citric acid metabolism from the other plant species studied. Their most striking feature was that the quantities of acid produced in healthy and in affected plants were not markedly different except at the beginning of the growing season and again near the end. But at these times the acid content of diseased plants was somewhat the greater. It may be noted in accounting for these slight differences that increase in soluble calcium in the sap occurs coincidentally with increase in citric acid in grape. This observation will be given further consideration in a later report.

Since the methods for determining isocitric acid (Pucher[7]), and Pucher and Vickery[8]) were unknown to the writer when the present investigations were in progress it is still not known whether this acid is among the organic acids produced by grape. But as will be indicated later, tartaric acid constitutes an important portion of the organic acid content of grape.

Malic acid.

The writer's previous studies (I l j i n [1]) [2]) [4]) [5])) have shown that the concentration of malic acid in plant sap is closely correlated with the concentration of soluble calcium. For example, white lupine grown in pots with such differing amounts of added lime as 18.1, 34.7, 38.7, and 70.8 g, were found to have 52, 116, 129, and 222 mg of malic acid, giving approximate ratios of added lime to malic acid of 1 : 3. The evidence of whether relationship of lime to malic acid is maintained in chlorotic plants and whether the alterations of malic acid metabolism in such affected plants tends toward increase or toward decrease is contradictory. In a series of analysis of the leaves of Noble apple made in 1943, malic acid in affected trees increased gradually to mid-summer and then gradually declined toward autumn. In comparison the concentration of this acid in healthy trees declined sharply at the beginning of the season and remained quite uniform thereafter. At all times there was from one-third to one-half as much malic acid in normal foliage as in chlorotic foliage. The reverse was the case in severely chlorotic trees however, since it was found that their malic acid content was approximately half that of normal ones. Similar comparative results were secured with cherry foliage and also with that of *Fraxinus excelsior*.

From the analytic data, as a whole, considering all the species tested, it must be concluded that malic acid production is not known to be closely correlated with alterations incident to lime-induced chlorosis.

Tartaric acid.

Since a large proportion of the organic acid content of grape is well known to be tartaric acid, special consideration was given to diseased and healthy plants of this species. As a result it was found that tartaric acid increases from 20–30 mg per g of dry weight in spring to 60–70 mg by midsummer and to 80–90 mg by early autumn. Meanwhile there is a correlated decrease in the proportion of soluble calcium in the sap, about 75% being in soluble form in spring and only 15% in autumn. Total tartaric acid metabolism in all other species of diseased plants, in general, was only 10–20% greater at any time than that in healthy ones.

Total organic acids.

As previously indicated, comparative studies of the organic acid

content of normal and diseased leaves of the several species of plants included determination of their total organic acids. This consisted of their identified acids such as citric, malic, and tartaric, and in some cases of an unidentified fraction. As regards total organic acids representative data from certain species are assembled in Table III.

TABLE III

Comparison of total organic acids in normal and chlorotic leaves, when sampled at succeeding intervals of approximaty 4 weeks

Kind of Plant	Condition	Total acid at successive intervals, m eq/1g of dry weight
Syringa vulgaris	Normal	0.73 — 1.03 — 1.23
	Diseased	0.93 — 1.18 — 1.75
Ailanthus glandulosa	Normal	0.89 — 1.17 — 1.55 — 0.87
	Diseased	1.03 — 1.49 — 1.96 — 1.56
Sambucus nigra	Normal	0.55 — 0.92 — 1.11 — 0.77
	Diseased	1.14 — 1.90 — 2.15 — 2.40
Grape (Rheinriesling)	Normal	0.60 — 1.05 — 0.91 — 0.60
	Diseased	0.75 — 1.50 — 1.04 — 0.73
Apple (Noble)	Normal	0.66 — 0.34 — 0.26 — 0.54
	Diseased	1.35 — 1.06 — 1.02 — 1.47
Cherry	Normal	0.74 — 1.16 — 1.12 — 1.22
	Diseased	1.12 — 1.31 — 1.34 — 1.23

It is to be noted from Table III, that the total organic acid content of the leaves of chlorotic woody plants always exceeds that of healthy ones. The differences at time of any given sampling may not be large but are consistently and sufficiently greater to warrant the conclusion that the metabolism of organic acids in sum total is enhanced in plants affected with lime-induced chlorosis.

The amount of unidentified organic acid was derived by difference between total organic acids and the sum of citric acid and malic acid together. Certain of the data on this constituent of the total organic acid content, which indicate the range in amount metabolized, are shown in Table IV.

As anticipated, different quantities of unidentified organic acids occur in different species, and furthermore the amounts of these acids present varies throughout the season. That variation in proportion of organic acids is also modified by the course of chlorosis is to be expected. This fact is best demonstrated by selecting from the data involving different species those secured by analyses of apple leaves, as shown in Table V.

TABLE IV

Amounts of unidentified organic acids in the leaves of certain plants		
Kinds of Plants	Acids	Serial analyses, results as m eq/1g of dry weight
Lupinus albus	total acid	1.60 — 2.08 — 2.30 — 2.48
	citric + malic	1.44 — 1.51 — 0.94 — 1.61
	unidentified	0.16 — 0.57 — 1.36 — 0.87
Syringa vulgaris	total acid	0.93 — 1.26 — 1.75 — 1.06
	citric + malic	0.74 — 1.28 — 1.27 — 0.77
	unidentified	0.19 — — — 0.38 — 0.29
Ailanthus glandulosa	total acid	1.05 — 1.49 — 1.96 — 1.56
	citric + malic	1.07 — 1.12 — 1.63 — 1.57
	unidentified	— — 0.37 — 0.33 — —

The data in Table V indicate that unidentified kinds of organic acids are metabolized in differing amounts depending upon the severity of the disease and upon the period when the samples are taken for analysis. It might be added, from analyses of normal and chlorotic cherry leaves as well as from other plant species, that the evidence secured verifies the conclusions provided by data in Table V.

TABLE V

Variation in total amounts of unidentified kinds of organic acids in apple leaves as correlated with severity of lime-induced chlorosis	
Condition	Difference between total acid and the sum of citric and malic acids at intervals, m eq/1g of dry weight
Normal	0.06 — 0.02 — 0.00 — 0.10 — 0.22
Slightly chlorotic	0.58 — 0.45 — 0.31 — 0.36 — 0.78
Severely chlorotic	0.83 — 0.68 — 0.52 — 0.48 — 0.73

Carbohydrates.

As a guide to the making of comparative analyses, series of determinations of sugars and starch were made, using normal leaves of *Sambucus nigra*, *Syringa vulgaris*, *Prunus avium*, the several varieties of grape, and the several varieties of apple previously mentioned. The results of these analyses showed that the periods of greatest change do not occur at the same times in different years, presumably because of different weather conditions. The direction of change of carbohydrate content of all species is the same, however, at any particular period of any year. With this basic information in hand, attention was given to modifications induced in plants

affected with lime-induced chlorosis in comparison with normal plants.

It was anticipated at the outset that carbohydrate metabolism would be altered in direct proportion to the degree of loss of the green pigments. The data, however, do not reflect such a simple relationship. The results of analyses of total carbohydrates of Noble and Bellflower apple, for example, are shown in Table VI.

TABLE VI

Comparative changes in total carbohydrate content of normal and chlorotic apples during the period May 11 to October 5

Kind	Condition	Successive intervals and mg of carbohydrate per g of dry weight
Noble . .	Normal	100 — 89 — 88 — 67 — 66 — 82 — 81
	Chlorotic	61 — 64 — 59 — 58 — 66 — 90 — 78
Bellflower	Normal	115 — 88 — 88 — 90 — 72 — 73 — 118
	Chlorotic	68 — 92 — 80 — 66 — 91 — 114 — 110

It should be explained that the intervals between successive samplings were approximately three weeks. The most obvious difference portrayed in Table VI is that which occurs during the early part of the growing season. During this period both photosynthesis and translocation from reserves in the woody tissues may account for the larger amount of carbohydrates present in leaves.

Similar findings were obtained with Rheinriesling grape foliage. The analytic data in this instance are given to show separately the starch content and the sugar content. Such data are assembled in Table VII.

TABLE VII

Comparison of changes in sugar and starch content of normal and chlorotic grape foliage during the period from June 8 to October 5

Condition	Constituent	Successive intervals and mg of constituent per g of dry weight
Normal	Sugar	29 — 55 — 70 — 93 — 34 — 47 — 62
Chlorotic	,,	3 — 9 — 40 — 93 — 63 — 80 — 99
Normal	Starch	20 — 27 — 30 — 36 — 4 — 7 — 12
Chlorotic	,,	21 — 26 — 28 — 25 — 27 — 22 — 4

Successive samplings for the data in Table VII, were made at average intervals of three weeks. As was found with apples, the carbohydrate content of normal grape foliage is the greater at the beginning of the growing season. During midsummer the carbo-

hydrates of normal plants are being translocated in appreciable quantities to the developing fruits and to points of storage in the trunks and limbs whereas in chlorotic plants, which were not bearing fruit, the carbohydrates tend to remain within the leaves. Among other species used to compare the total sugar and starch contents of leaves of normal plants with those of chlorotic ones were *Prunus avium, Syringa vulgaris, Sambucus nigra, Robinia pseudacacia, Ailanthus glandulosa, Fraxinus excelsior,* and species of lupines. The type of changes in all followed a similar pattern. As evidence, comparisons are shown in Table VIII, which follows, showing analytic results from three of these species.

TABLE VIII

Results of analyses of sugar and starch content of foliage of certain normal and chlorotic species of woody plants

Plant species	Condition	Content of sugar and starch mg per 1 g of dry weight	
Syringa vulgaris	Normal	Sugar	54 — 39 — 55
	Chlorotic	,,	47 — 23 — 12
	Normal	Starch	43 — 19 — 33
	Chlorotic	,,	23 — 23 — 13
Sambucus nigra	Normal	Sugar	55 — 35 — 48 — 69
	Chlorotic	,,	34 — 35 — 39 — 25
	Normal	Starch	36 — 37 — 55 — 41
	Chlorotic	,,	16 — 21 — 25 — 20
Fraxinus excelsior . . .	Normal	Sugar	40 — 28 — 33
	Chlorotic	,,	50 — 20 — 25
	Normal	Starch	56 — 17 — 34
	Chlorotic	,,	36 — 20 — 24

Manifestly the elaboration of sugar and starch in trees that are affected with lime-induced chlorosis, judging from the analytic results of leaves, appears to proceed at a fairly high rate. Yet the carbohydrate reserves in storage must gradually become impoverished or at least become quite exhausted, for eventually such chlorotic trees succumb.

SUMMARY

This study is concerned with changes in organic acids and carbohydrates in the foliage of plants affected with lime-induced chlorosis. The saps of seventeen species of plants were analyzed during a period of four seasons. Determinations were made of total organic acid content, and also of certain constituent acids such as citric, malic, and tartaric. In some instances a

residue of unidentified acids was determined by difference between total acids and identified constituent acids.

In such species as metabolize citric acid, several times more citric acid may occur in the leaves of chlorotic plants than in healthy ones, the amount present being directly correlated with the severity of the disease. Even before symptoms of chlorosis are visible, large increases in citric acid may have taken place.

As might be anticipated, the amount of citric acid metabolized varies throughout the growing season. In healthy foliage the smallest amounts are present at the beginning of the growing season and the largest amounts in autumn. In affected foliage, however, the maximum concentrations are found in spring.

The metabolism of malic acid and tartaric acid is modified to a less marked degree than that of citric acid in plants affected with lime-induced chlorosis.

The content of unidentified acids is largest in the most severely chlorotic leaves.

The metabolism of organic acids seems to be directly correlated with the concentration of soluble calcium in the sap, a relationship to be given special consideration in a subsequent report.

Analyses reveal that the carbohydrate content of diseased foliage in some species may exceed that in healthy foliage in spite of the changes that involve chlorophyll. Apparently this phenomenon is related partly to impairment of translocation of carbohydrates. Carbohydrates are largely translocated from leaves to places of storage in fruits and woody tissues of normal plants, whereas in chlorotic plants they tend to remain in the foliage.

In general, the course of metabolic processes involving organic acids and carbohydrates is unbalanced in lime-induced chlorosis.

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LITERATURE CITED

1) Iljin, W. S., Precipitation of calcium by plants in different habitats. Bull. Assoc Russ. Sci. (Prague) **5**, 37–66 (1937).
2) Iljin, W. S., Calcium content in different plants and its influence on production of organic acids. Bull. Assoc. Russ. Sci. (Prague) **7**, 43–76 (1938).
3) Iljin, W. S., Quantitative microanalysis of salts and organic acids in plants. Bull. Assoc. Russ. Sci. (Prague) **8**, 48–94 (1939).
4) Iljin, W. S., Boden und Pflanzen. II. Physiologie und Biochemie der Kalk- und Kieselpflanzen. Bull. Assoc. Russ. Sci. (Prague) **10**, 75–116 (1940).
5) Iljin, W. S., Salze und organische Säuren bei Kalkpflanzen. Flora **37**, 265–299 (1944).
6) Iljin, W. S., Metabolism of plants affected with lime-induced chlorosis (Calciose). I. Nitrogen metabolism. Plant and Soil **3**, 239–256 (1951).
7) Pucher, G. W., The organic acids of the leaves of *Bryophyllum calycinum*. J. Biol. Chem. **145**, 511–523 (1942).
8) Pucher, G. W. and Vickery, H. B. On the identity of the so called crassulacean malic acid with isocitric acid. J. Biol. Chem. **145**, 525–532 (1942).

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RAPID METHODS FOR DETERMINING DIFFERENT TYPES OF SULPHUR COMPOUNDS IN SOIL

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I. INTRODUCTION

For investigating the sulphur cycle in freshly reclaimed or inundated soil, it is of great importance to have rapid and reliable methods available to determine the quantity of various types of sulphur compounds that may occur in soil. Because sulphuric acid frequently forms after aeration of anaerobic soil, it is particularly desirable to determine the quantity of sulphur compounds that may be oxidized to sulphuric acid (total oxidizable sulphur). For this reason the laboratory of the Zuidersea Reclamation Works asked the Research Laboratory of N.V. De Bataafsche Petroleum Maatschappij some years ago to give advice regarding the most suitable method for rapid determination of sulphides, polisulphides and sulphates in soil. On the strength of a limited number of preliminary experiments, three different methods were suggested for converting these three types of sulphur compounds into hydrogen sulphide; this gas could then be measured iodometrically via cadmium- or zinc-sulphide as an intermediate step. This advice was followed and the methods, extended somewhat by the laboratory of the Zuidersea Reclamation Works, proved to be suitable for examining hundreds of soil samples. A description of these extended methods is given below, together with a few general remarks concerning their suitability. The results obtained will be stated in later communications.

II. GENERAL PRINCIPLES

We may assume that sulphur in soil may occur in the following forms: sulphides (mainly iron sulphide), polysulphides (mainly

pyrite), sulphites, thiosulphates, sulphates, elementary (free) sulphur and organically bound sulphur. As a rule these compounds are determined quantitatively, according to the methods usually applied in soil studies, by treating them with various extracting and oxidizing agents and converting them into sulphates, which are then determined gravimetrically or titrimetrically (see for instance v. d. S p e k [5]).

To avoid the cumbersome gravimetric and the somewhat unreliable titrimetric sulphate determinations we preferred to convert all sulphur compounds into hydrogen sulphide, which can be determined iodometrically either directly or indirectly via some insoluble sulphide, e.g. cadmium sulphide.

A differentiation between various groups of sulphur compounds may then be obtained by noting their response to different means for their conversion into hydrogen sulphide. A number of sulphur compounds, viz. acid-soluble sulphides, release their sulphur as hydrogen sulphide on being boiled with hydrochloric acid. Others, such as pyrite, some other "polysulphides", and elementary sulphur, are converted into hydrogen sulphide by reduction with tin and hydrochloric acid [1]). For converting sulphates a more drastic attack is necessary, e.g. ignition with *ferrum reductum* [2]), followed by boiling of the reaction products with hydrochloric acid.

Direct determination of the liberated hydrogen sulphide, for instance, by bubbling through a known quantity of iodine solution and back-titrating unused iodine with sodium thiosulphate, is unreliable in the presence of other gases that reduce iodine, such as SO_2. We therefore preferred to precipitate the hydrogen sulphide as cadmium sulphide (or partly as zinc sulphide), followed by treatment of this precipitate with hydrochloric acid in the presence of a known quantity of iodine and back-titration of the unused iodine with thiosulphate [3]).

The three methods mentioned above may be considered to represent sulphidic sulphur, total oxidizable sulphur and total sulphur respectively. From them polysulphidic and free sulphur and also sulphate sulphur may be calculated by successive subtraction. As a supplement to these three methods, the quantity of "free sulphur" (or rather "extractable sulphur") might be determined separately by extraction with a suitable solvent such as chloroform or acetone.

Our main attention, however, was devoted to the tin-reduction method, as the sulphur content found in this way may be considered to represent the total quantity of oxidizable sulphur compounds.

III. EXPERIMENTAL

A. *Determination of monosulphidic sulphur compounds.*

The content of monosulphidic sulphur compounds (determined by boiling the sample with hydrochloric acid 1 : 1 for two or three hours while driving out the gaseous reaction-products with oxygen-free nitrogen via a wash-bottle with water into a solution of cadmium acetate and zinc acetate, subsequent addition of iodine solution and hydrochloric acid and back-titration of unused iodine) was invariably found negligible in well-aerated soils, but in anaerobic sea- muds it sometimes amounted to 0.45% (percentage by weight of sulphur calculated on the dried sample — all other percentages given are also on this basis).

When, however, we collected the gaseous products directly in an iodine solution, followed by direct back-titration of the unused iodine without the intermediate use of cadmium sulphide, as was done by Verhoop[4]), we nearly always found a considerably higher sulphur content. The same high value was obtained when the gases were passed through a hydrogen peroxide–ammonia solution with subsequent gravimetric determination of barium sulphate. This higher result was probably caused by SO_2 being produced in addition to H_2S. This SO_2 might have been formed through reaction of the soil oxidants with the H_2S liberated or through reaction of hydrochloric acid with any sulphites or thiosulphates present. (Perhaps it is possible to determine the content of sulphites and thiosulphates from the difference between the results of these methods; but this point was not further investigated).

B. *Determination of the total quantity of oxidizable sulphur.*

The apparatus described for this method by Treadwell[1]) had to be enlarged to accomodate the rather voluminous soil samples. The investigation was started on a macro-scale and was further developed on a semimicro-scale (see section IV).

Reduction of pyrite and other polysulphides in soil by means of tin and hydrochloric acid is a rather slow reaction. However, there was

always a definite end-point, which could be checked by repeated addition of fresh tin and hydrochloric acid and continued boiling. Sulphates are not reduced by this method. Here, too, the liberated hydrogen sulphide is determined iodometrically via cadmium sulphide.

The procedure described in detail in Section IV gave reproducible results. It is assumed that they represent the total quantity of oxidizable sulphur (monosulphides, polysulphides, sulphites, thiosulphates, free and organically bound sulphur). This total quantity of oxidizable sulphur was examined in a great number of soil samples, both aerobic and anaerobic. The values found varied from none to about 1.5%.

C. *Determination of extractable sulphur.*

To get some idea as to what part of the sulphur content found with the tin reduction method is due to inorganically bound sulphur on the one hand and what part to free and organically bound sulphur on the other, we made a few extractions with acetone to remove the latter group. It is an open question whether all the free sulphur and all the organically bound sulphur actually dissolve in acetone. However, other solvents such as carbontetrachloride and carbon disulphide were unsuitable because of their sulphur content.

In one soil sample, the quantities of oxidizable sulphur determined with the tin-reduction method before and after extraction with acetone were 1.24 and 1.07% respectively, which shows that not all the sulphur found by tin-reduction was inorganically bound sulphur. This method was not further developed, however, because content of extractable sulphur was not of primary importance for our investigation.

D. *Determination of the total sulphur content.*

For this purpose Gröger's [2]) method (ignition with *ferrum reductum* and subsequent boiling of the reaction products with hydrochloric acid) followed by determination of the hydrogen sulphide via cadmium sulphide, was very suitable. A number of analyses were made of the total sulphur content of the same sample according to this method and also gravimetrically as barium sulphate according to the conventional Fresenius method.

Example of results:

Gröger's method, 2.22 and 2.23%;

Fresenius' method, 2.14 and 2.19%.

With a few soil samples examined later, which had a very high content of basic ferric sulphate, a larger quantity of *ferrum reductum* was necessary for the quantity of soil examined. Although this indicates that the procedure should be applied critically, Gröger's method is, in our opinion, a very attractive one for rapid determinations of total sulphur content.

IV. RECOMMANDED EXPERIMENTAL PROCEDURES

The apparatus used for all the methods is represented in the attached figure.

Method A: Determination of the quantity of monosulphidic sulphur.

1. A weighed quantity of soil (about 4 g) is introduced in its original state (not dried) into the Kjeldahl flask *A* and mixed with 10 ml of

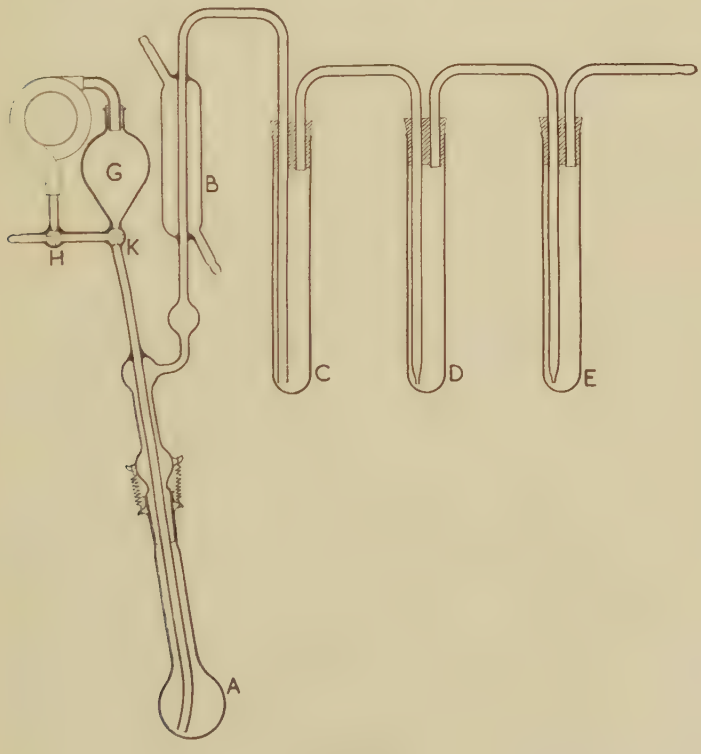

Fig. 1

boiled-out distilled water. Tube *C* is also filled with pure water. Tubes *D* and *E* are filled with 50 and 25 ml, respectively, of a cadmium-zinc acetate solution (5 g cadmium acetate, 20 g zinc acetate and 250 ml glacial acetic acid per liter). Funnel *G* is filled with hydrochloric acid 1 : 1. Through the three-way cocks *H* and *K*, oxygen-free nitrogen is bubbled untill al the air is removed from the apparatus. Then 20 ml of hydrochloric acid 1 : 1 is added dropwise to the soil suspension from funnel *G* under nitrogen pressure and the contents of flask *A* are boiled for two hours. Subsequently another 20 ml of hydrochloric acid 1 : 1 is added under nitrogen pressure and the contents are boiled for another hour. The hydrogen sulphide dissolved in the water of tube *C* is then removed by boiling the contents of this tube for 5 minutes. During all these manipulations the nitrogen current must not be interrupted. The solutions in *D* and *E* are now mixed and, after addition of 10 ml of 0.1 N iodine solution, the liquid is acidified with 20 ml of hydrochloric acid 1 : 1 and diluted with 100 ml of water. The remaining quantity of iodine is back-titrated with 0.1 N sodium thiosulphate solution.

2. A similar procedure can be applied if one wants to collect the hydrogen sulphide directly in iodine solution, with subsequent back-titration of unused iodine (method A_2). The result of this analysis may be considerably higher than with method A_1, indicating the presence of other oxidisable gases, such as SO_2.

Method B: Determination of the total quantity of oxidisable sulphur.

A weighed quantity of the soil sample (dried if necessary; dry weight about 200 mg) is introduced into flask *A* and mixed with about 5 g of tin powder (to introduce the small quantity of soil into the flask quantitatively it is recommended that a piece of tin-foil paper be used for weighing and put into the flask together with the soil). After the air has been removed from the apparatus by means of nitrogen, 5 ml of hydrochloric acid (concentrated, spec. grav. 1.19) is added from the funnel the contents of the flask are boiled gently for an hour, mixed with another 5 ml of concentrated hydrochloric acid, boiled for another hour, mixed with 10 ml or hydrochloric acid and after boiling again for another hour the contents of tube *B* are also boiled for 5 minutes. Throughout these manipulations nitrogen is passed through. In other respects the procedure is identical with that of method A_1.

Method C: Determination of the quantity of extractable sulphur.

After the sample has been dried (preferably in a vacuum desiccator over P_2O_5 or in an oxygen-free nitrogen current at 40°C, to avoid oxidation of the sulphur compounds), a quantity of 50 100 g is extracted with acetone for 10 hours at room temperature until after evaporation of the acetone no residue is left. Before and after this extraction the tin reduction method *B* is applied to part of the sample.

Method D: Determination of the total sulphur content.

A small reaction tube of combustion glass is filled with a homogenious mixture of one part by weight of a dried soil sample and three parts by weight of iron powder (heated beforehand to a dull red heat in a hydrogen current). This mixture is covered with one extra part by weight of iron powder. The tube is suspended in a pierced asbestos plate over a Bunsen burner and while oxygen-free nitrogen is passed through (for instance, with the help of a porcelain tube of a Rose crucible) it is heated, at first gently and finally for 10 minutes to a dull red heat. After being cooled in a nitrogen current, the tube with the contents is introduced into flask *A* of the apparatus in the figure and the content of iron sulphide is determined according to method A_1. For sulphate-rich samples it is desirable to change ratio of soil to iron powder from 1 : 3 to 1 : 4 or even 1 : 5.

V. SUMMARY OF RESULTS

By means of the methods discussed it seems to be possible to distinguish at least five types of sulphur compounds in soil, although sometimes the description may be somewhat arbitrary. See Table I.

TABLE I

Survey of the methods discussed, indicating the types of sulphur compounds determined in each method

Method	Sulphides	Sulphites and Thiosulphates	Polysulphides	Free and organically bound sulphur	Sulphates
A_1					
A_2	+	+	—	—	—
B	"	"	"	"	"
C	—	—	—	+	—
D	+	+	+	+	+

Methods A_2 and D have their equivalent in more laborious gravimetric methods for determining sulphates that give practically the same results (see for instance, v. d. Spek [5]).

In practice method B has proved to be particularly valuable; it is comparatively rapid and its results represent the quantity of sulphur compounds that may form sulphiric acid on aeration. Subtraction of the sulphur content found with methods A_2 and C leaves what may be described as mainly pyrite sulphur. Formerly, this pyrite value could only be calculated from the difference between the sulphate contents of the hydrochloric acid extract and the aqua regia extract of the soil sample, which is questionable and highly time-consuming.

Although method D should be applied with some care, it, too, has definite advantages in replacing the more laborious gravimetric Fresenius' method.

SUMMARY

A description is given of methods for determining quantitatively the total sulphur, oxidizable sulphur, monosulphidic sulphur, and extractable sulphur (free and organically bound) in soil. Monosulphidic sulphur is determined by boiling the soil sample with hydrochloric acid and distilling the liberated hydrogen sulphide with a nitrogen current into a cadmium acetate solution, after which the cadmium sulphide precipitate is determined iodometrically. Oxidizable sulphur is determined in a similar way by exposing the sample to tin and concentrated hydrochloric acid. Total sulphur is also determined via hydrogen sulphide, obtained in this case by ignition of the sample with *ferrum reductum* in a nitrogen current and liberation of hydrogen sulphide with hydrochloric acid. For determining extractable sulphur, acetone is recommanded.

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REFERENCES

1) Treadwell, F. P., Beitrag zur Schwefelbestimmung II. Ber. deut. chem. Ges. **25**, 2377 (1892).

2) Gröger cited by Treadwell, F. P., in: Beitrag zur quantitatieven Bestimmung des Schwefels. Ber. deut. chem. Ges. **24**, 1937 (1891).

3) Scott, W. W., Standard methods of chemical analysis, 5th edition, Vol. I, p. 912.

4) Verhoop, J. A. W., Chemische en microbiologische omzettingen van ijzersulphiden in den bodem (Chemical and microbiological transformations of iron sulphides in soil). Thesis Leiden-Wageningen, (1940).

5) Spek, J. van der, Bijdrage tot de kennis van de zure gronden van het Nederlandsch alluvium (Contribution to the knowledge of the acid soils of the Dutch alluvium). Verslagen Landbouwk. Onderzoek. No. 40B, (1934).

Plant and Soil III, no 4 October 1951

BIOSYNTHESIS OF AMINO ACIDS ACCORDING TO SOIL FERTILITY

II. METHIONINE CONTENT OF PLANTS AND THE SULFUR APPLIED †)

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The organic composition of plants and their quality as feeds and foods vary according to the differences in the fertility of the soils growing them [3]) §). The synthesis of amino acids composing the proteins has been considered as occurring in the green leaf. It is by enzymes that the carbohydrates, of photosynthetic origin, are converted into the constituents of protein. Since the inorganic nutrient elements from the soil may serve not only as enzymes but also as portions of the prosthetic groups and as atoms in the final molecular products, that fraction of the soil deserves more consideration. Sulfur was given attention and preliminary study in connection with the plants' contents of methionine, the sulfurcarrying amino acid.

PREVIOUS WORK

Aside from occurring in the amino acids cystine, cysteine, homocysteine and methionine, the soil-borne sulfur goes into both of the vitamins biotin and thiamine. Amino acids (presumably those devoid of sulfur) amides, ammonia, and acid-hydrolyzable carbohydrate accumulate in mustard [1]) when the sulfur is offered in low supply.

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§) Figure in parenthesis refers to literature cited.

Using sulfur-deficient tomato plants, Nightingale[2]) found them extremely high in carbohydrates and with much more nitrate than normal. More recent investigations indicate that fat, rather than carbohydrate, may accumulate when sulfur is in low supply. Tisdale *et al*[6]) reported that the methionine and cystine contents of two strains of alfalfa indicated that the sulfate in the substrate influenced the concentration of these two organic acids in the alfalfa forage. A metabolic block in biosynthesis causing a "backlog" of other undetermined components would indicate that some sulfur structure might be limiting the formation of the protoplasmic protein.

Since methionine, one of the essential amino acids and one containing sulfur, is often in low supply in legumes, the study reported here was undertaken. It was planned to determine the methionine contents of some of the plants and its variation in concentration there when varied quantities of sulfur were available in the soil or in a more carefully controlled medium. The technique used for measuring methionine was the micro-biological procedure of Stokes and Gunness[4]) [5]), using *Streptococcus faecalis* R9790 as the assay organism.

EXPERIMENTAL RESULTS

Alfalfa and soybean plants were first grown in sand cultures using appropriate nutrient solutions containing variable amounts of sulfate. The striking influence of the sulfur supply on the concentration of methionine in dry matter of these plants is shown in Table I. The increase in concentration of this amino acid went up as more sulfate was offered, in the nutrient medium for several increments and then went down with the highest sulfate offerings.

TABLE I

Methionine concentration (mg/g) in alfalfa and soybean hays according to the amounts of sulfur offered in the nutrient solution

Sulfur Offered P.P.M.	Alfalfa mg/g	Soybeans mg/g
0	1.96	2.67
16	4.50	3.08
32	5.01	3.56
64	5.37	—
96	4.61	6.41
128	2.70	5.90

In a field trial using a more highly weathered soil for alfalfa, the applications of magnesium sulfate as fertilizer treatments brought increase of the concentration of methionine again, Table II. The addition of barnyard manure along with the sulfur, in the second year of these tests, brought even greater concentrations of this amino acid in the alfalfa.

TABLE II

Methionine concentration (mg/g) in alfalfa hays according to the sulfur applied to the soil

Treatment	Methionine		
	6/24/48	7/31/48	4/11/49
None	3.10	3.21	2.91
100 lbs/acre $MgSO_4$	3.41	3.64	5.87 *)

*) 10 tons per acre of barnyard manure applied.

In an effort to substantiate further these general findings, some flowers of sulfur were applied at the rate of 200 pounds per acre on an adjoining field where the phosphate requirements according to soil tests had been previously met by use of rock phosphate to exclude the sulfur. This simple soil treatment with sulfur doubled the concentration of methionine in the Sudan grass, the crop used in this test. It is significant to note that the concentration of nitrogen as total was not increased, Table III. If the constancy of amino nitrogen holds, then methionine was made by sulfur additions but at the expense of some other amino acids. The inventory of the other amino acids verified this concept. Increasing the sulfur additions by supplementing the flowers of it with magnesium sulfate, lowered the methionine concentration, and at the same time a lowered total nitrogen also resulted.

TABLE III

Methionine concentration (mg/g) in Sudan grass (dried) with and without sulfur and with magnesium on soil treated with rock phosphate

Treatment	Total nitrogen (%)	Methionine (mg/g)
1. No sulfur applied	2.20	2.04
2. 200 lbs sulfur per acre	2.44	4.06
3. 200 lbs sulfur per acre + 100 lbs per acre $MgSO_4$	1.96	1.53

DISCUSSION

Since methionine is the one of three sulfur-containing amino acids considered essential in nutrition of the rat, and, since it is also commonly deficient in feeds to call for supplements providing it, these data suggest that the proteins have probably been more complete as a result of the sulfate carried along unwittingly in superphosphate. Likewise there is the suggestion that growing legumes for the sake of nitrogen and considering it the index of proteins has fallen short of providing complete protein for animal nutrition unless sulfur was amply present in the soil. Soil treatments with sulfur, according to the tests here reported, suggest themselves as means of providing methionine of high concentrations in the grasses. These forages, then, can produce more complete proteins, at least with reference to methionine often deficient in peas, beans, peanuts and even in casein for fuller nutrition, provided the soil fertility offers the essentials for the synthesis of them by the plant. These synthetic processes may use fertility elements not only as catalysts or intermediates in the metabolic reactions but as constituent parts of the resulting organic compounds much as sulfur suggests itself in case of these results for methionine.

Plants serve in the larger cycle of nutrition of microbes, animals and man, not as deliverers of ash elements or inorganic essentials only, but as biosynthetic creators, for example, of methionine and other essential amino acids according to the fertility of the soil. Conversely, then, the precise diagnostic techniques using any biological assay of soil fertility must measure molecules as the products of biosynthesis rather than determine only the quantity of the soil-borne inorganic elements carried along in these complicated reactions and interactions. This type of thinking reduces the stature of the entire biotic pyramid to the common denominator of the bio-colloids uniting with enzymatic specifity to yield either construction or destruction of life according to the inorganic elements coming from the soil.

The diversity of the methionine concentrations in the crops studied so far points out that the fertility of the soil plays the major role in agricultural production as this can be protein output. That sulfur unwittingly put into the soil as sulfates — in company with many other fertilizer elements taking full credit for the measurable effects

on the plants — should reflect its help in protein synthesis and thereby cell multiplication, brings this concept into sharper focus. Here is a new emphasis, via an amino acid widely deficient in feeds and foods, on the creative functions of the soil.

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REFERENCES

1. Eaton, Scott, Sulfur content of seeds and seed weight in relation to effects of sulfur deficiency on growth of sunflower plants. Plant Physiol. **17**, 422–434 (1942).
2. Nightingale, G. T., Schermerhorn, L. G. and Robbins, W. R., Effects of sulfur deficiency on metabolism in tomato. Plant Physiol. **7**, 565–596 (1932).
3. Sheldon, V. L., Blue, Wm. G. and Albrecht, Wm. A., Diversity of amino acids in legumes according to the soil fertility. Science **108**, 426–428 (1948).
4. Stokes, J. L. and Gunness, M., Microbiological method for determination of amino acids I: Aspartic acid and serine. J. Biol. Chem. **157**, 651–659 (1945).
5. Stokes, J. L., Gunness, M., Dwyer, I. M. and Caswell, M. C., Microbiological methods for the determination of amino acids II: A uniform assay procedure for the ten essential amino acids. J. Biol. Chem. **160**, 35–49 (1945).
6. Tisdale, S. L., Davis, R. L., Kingsley, A. F. and Mertz, E. R., Methionine and cystine contents of two strains of alfalfa as inlfuenced by different concentrations of the sulfate ion. J. Am. Soc. Agron. **42**, 221–225 (1950).

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THE USE OF *ASPERGILLUS NIGER* (VAN TIEGH.) M STRAIN AS A TEST ORGANISM IN THE STUDY OF MOLYBDENUM AS A PLANT NUTRIENT

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PART ONE

GENERAL TECHNIQUE AND EVALUATION OF METHODS FOR THE ELIMINATION OF MOLYBDENUM

The use of living material, in this instance a micro-organism, for the biological assay of organic or mineral materials depends on numerous assumptions. It is beyond the scope of this paper to review the principles of bioassay which comprise aspects of great complexity. It will be sufficient to outline the reasons for adopting this procedure and the fundamental requirements in the reliable application of the method.

It was shown by Steinberg (1936, 1937, 1939) [28] [29] [30] and later widely confirmed that strains of the fungus *Aspergillus niger* are unable to make appreciable growth in certain media when deprived of molybdenum (*sensu lato*), or given concentrations below certain known limits. The sensitivity of the fungus to low levels of molybdenum exceeds any chemical test yet devised. Mulder (1948*b*) [17] and Gerretsen (1948) [4] have shown this for molybdenum and other trace elements. Thus the ultimate sensitivity of the dithiol reaction for molybdenum studied by Welcher (1947) [33] and Piper and Beckwith (1948) [24] might under favourable conditions extend to 0.05 μg Mo, but for reliable analytical procedure the extreme limit at present is probably 0.1—0.2 μg. The use of *A. niger* as a test organism for assay purposes is by no means new and earlier work in this field has been reviewed by Nicholas (1950) [18]. The application of the method to molybdenum was probably first suggested by Mulder (1939) [15] and applied by him (Mulder 1948*b*) [17] and Nicholas and Fielding (1948, 1950) [19] [20] to the assay of molyb-

denum in plant material and soils. The *A. niger* method described here will consistently detect concentrations of the order of 10^{-4} μg Mo — an extension of the chemical range by about 1000 fold. Whilst it is true that by taking sufficient of the test material the overall quantity of molybdenum might be brought within the scope of chemical or spectrographic methods, other factors, e.g. limited material, risk of contamination during concentration, difficulty of manipulation, time and expense, limit or prevent the application of this approach.

The validity of the assay technique requires (among other things) the following conditions: that the response to molybdenum is specific (i.e. that molybdenum cannot be replaced by any other element even at relatively much reduced efficiency or by an unsuspected organic constituent); that interference by relatively wide differences in the concentration of other materials (mineral elements especially) is small, that day-to-day changes due to inoculum, saltation, climate, or other factors, are either unimportant, non-existent, or compensated, and that *total* extraneous sources of molybdenum are consistently excluded by the technique to the extent of about 99.5% of the upper limit of the range of assay, (here regarded as 0.01 μg Mo). This is important since all determinations are in fact recorded as the *difference* between their true value and an assumed "nil" which must therefore be as low as possible. Thus accuracy is greater when the unknown concentration is large compared with the "nil" but sensitivity is correspondingly reduced as the former approximates to the upper limit of the range. Medium values of the range are therefore to be preferred where possible.

The work described in this paper was undertaken to test the factors determining consistently efficient removal of molybdenum contamination in reagents, water and other culture materials and to develop a reliable procedure for detection and estimation of such contamination.

EXPERIMENTAL

As much of this paper is concerned with the development, step by step of a technique tested at each stage, the data relative to each factor are more conveniently introduced *seriatim* than in the more orthodox fashion in a section devoted to "results" as opposed to "methods". The work has been carried out between 1947 and 1951. The effects of variations in technique, particular purification procedures, sources of contamination, or error, are dealt with mainly in order of their relevance or interest. Application to the determination of molybdenum in culture and plant materials, and the biochemical aspects of molybdenum nutrition are considered in part two and in papers now in preparation.

Strain.

In all the work the species *Aspergillus niger* (van Tiegh.) "M" strain derived from a single spore culture supplied through the generosity of Dr. E. G. Mulder of Groningen was used. The culture was maintained by regular subcultures from a spore suspension on agar slopes using Mulder's (1948) medium [17]) without addition of extra molybdenum *).

Glassware and culture vessels.

The standard culture flasks used were 500 ml conical Erlenmeyer flasks. As far as present experience allows, no distinction can be made between pyrex or hysil glass in this work, and either brand of borosilicate glass may be used indiscriminately. Steinberg (1936) [28]) concluded similarly that for molybdenum deficiency there was no significant distinction between pyrex glass and silica.

500 ml flasks were considered to be the best compromise between the surface area presented by the 50 ml of culture fluid used, and the space occupied in the incubator. In order to test the effect of size of flask, cultures were grown in 250 ml, 500 ml and 1000 ml flasks at "low" and "high" values of the standard concentration range (see below). The yields and surface areas of the cultures are shown in table I.

TABLE I

Effect of Culture Flask Capacity on Mycelial Yield

Culture flask Volume and Surface Area of Medium	Mo μg	Mean Yield (mg dry wt.)
250 ml 50.3 sq. cm	"nil"	22
	0.0003	85
	0.01	638 (*a*)
	0.01	593 (*b*)
500 ml 63.6 sq. cm	"nil"	55
	0.0003	73
	0.01	806 (*a*)
	0.01	876 (*b*)
1000 ml 95.1 sq. cm	"nil"	57
	0.01	898 (*b*)

The results indicated that there was an appreciable increase in yield up to 500 ml capacity but probably not beyond this, at the higher molybdenum levels.

*) Thanks are due to Mr. A. H. Fielding for maintaining and supplying the sporulating cultures used to prepare inocula.

As far as possible flasks used for standard growth series and known "low" concentrations of molybdenum were retained for their respective purposes. It was not always possible to ensure this precaution however, and the effect of using flasks for "low" levels after previous contamination by "high" levels was tested. The effect was not important so long as the following routine cleaning procedure (adopted for all glassware) was included before every set of determinations.

; Flasks were first rinsed thoroughly several times under a running tap and then washed with hot water and a detergent. The flasks were rinsed with distilled water and the necks inverted over a steaming device which accomodated 4—6 flasks at one time. The steaming comprised exposure to "steam" from rapidly boiling 20% hydrochloric acid issuing from a small jet for a period of 20 minutes. Eack flask was supported by a wide glass ring. After steaming, the flasks were washed down inside, and outside the neck, first with glass distilled water three times and then with twice glass distilled water. Care was taken to keep the outside dry after washing down to prevent drainage from re-entering or reaching the rim when inverting to drain them.

It was soon found that the orthodox cotton wool plugs used for mycological cultures introduced appreciable contamination (Plate No. 1). Small groups of spores and normal mycelium frequently developed in "nil" cultures directly under the neck of the flask. This trouble was satisfactorily eliminated by covering the open necks with inverted 50 ml squat beakers. The necessary air seal and ventilation was obtained by packing a ring of cotton wool in the annular space between the flask neck and the beaker, below the rim of the former. The beakers also were always cleaned as described above. Petri dishes were not employed in this work on account of cost, difficulty in cleaning satisfactorily and the fact that temperature variation in a stack of dishes probably outweighed the anticipated advantage from saving space.

Water.

The water used to make up and dilute culture media was obtained after distillation twice from pyrex glass stills with double splash traps (H e w i t t 1951) [6]), of water distilled in tinned copper stills with solid silver condensers. It was stored in pyrex glass jars, carboys or aspirators. Wash-bottles were provided with quickfit ground glass

jet assemblies fitting *outside* the necks of the flasks. The effects of using water from other sources is shown later in relation to the assay of water supplies. The twice glass distilled water was inferred from comparative tests to contain appreciably *less* than 0.000003 p.p.m. Mo.

Culture medium.

The culture medium was based on the formula of Mulder (1948*a*) [16]) with the addition of certain other microelements whose presence might ultimately prove to be essential. It was prepared at 5× normal concentration as shown below, before purification and was diluted just before use.

Glucose	250	g
KNO_3	25	g
$NaH_2PO_4 2H_2O$	12.5	g
$MgSO_4 7H_2O$	5	g
$Ca(NO_3)_2$ 2M. Solution	1	ml
Water	1000	ml

Micronutrient supplement added to each culture after dilution

Fe as $FeCl_3$	30	μg per 50 ml culture
Zn as $ZnSO_4$	20	μg ,, ,, ,,
Cu as $CuSO_4$	7.5	μg ,, ,, ,,
Mn as $MnSO_4$	5	μg ,, ,, ,,
Ga as $Ga(NO_3)_2$	5	μg ,, ,, ,,
Co as $CoSO_4$	1	μg ,, ,, ,,
Ni as $NiSO_4$	1	μg ,, ,, ,,

It will be noted that a small concentration of calcium ions is included in the formula above. This was done in case calcium might prove beneficial, and because many of the substances to be examined would contain calcium. No *significant* differences could be established for the yields in the presence of molybdenum with and without calcium, but in the presence of this element yields were consistently greater by about 8–20% than in its absence. Reduction of the phosphate level to one quarter had a corresponding depressive effect on yields and reduced sporulation.

After the addition to the flasks of the purified basal nutrients the micronutrient elements were added separately from stock solutions, at the rate of 0.1 ml of each per 50 ml of medium. Sterilisation was

carried out with the complete nutrient medium (except molybdenum) and the glucose was not sterilised separately.

Purification of nutrient reagents.

Before sterilisation the main constituents of the basal culture medium were purified to eliminate molybdenum. The effect of several procedures to achieve this end will be described in this section after outlining the standard method employed at present.

The principal salts, and glucose were obtained as A.R. grade materials, the latter "spectrographically molybdenum free" when available. In earlier work the glucose was recrystallised but the procedure was considered to be relatively extravagant and in later work this step was omitted. The salts were always recrystallised two or three times in large batches (1–2 kg). Recrystallisation was carried out from glass distilled water with the addition of an equal volume of purified alcohol as originally described by Hewitt (1947) [5]). This permitted yields of 90% or thereabouts for each step. It was found advisable to allow the crystallising liquor to cool to below 45–50°C before adding the alcohol as this avoided the formation of two phases which did not readily homogenise. Frequent stirring for the whole period of crystallisation was most necessary to prevent the formation of a hardcrystal "rock" in the beaker. The crystals were separated by filtration on a sintered filter beaker or on a carefully cleaned Buchner funnel and No. 50 Whatman paper. The crystals were washed with alcohol, dried in a current of air and finally at 30–35°C in an oven.

The process of recrystallisation eliminated a proportion of the molybdenum present but not the whole. The factors affecting the success of recrystallisation as a purification technique have been reviewed elsewhere by one of us (Hewitt 1951) [6]). The relative effectiveness here may be judged from the data below (table II) where the approximate molybdenum content of unrecrystallised and recrystallised salts is also shown. The tests were made by substituting separately each salt in turn in the completely purified medium by the unpurified ones as shown. It will be seen that effect of recrystallisation was not consistent and of little real value alone. Recrystallisation of sodium phosphate appeared to collect extraneous molybdenum. On the whole it was considered that preliminary recrystallisation was advantageous as a first stage.

The calcium nitrate solution of corresponding quality was prepared by neutralising pure "batch analysed reagent" HNO_3 (1 : 1 in water) with a thin slurry of A.R. $CaCO_3$ and filtering.

Each of the principal salts and glucose were then further purified according to the procedure based on a method mentioned (without details) by Stout and Meagher (1948) [32]) and used extensively for the routine purification of stock solutions required for the production of molybdenum deficiency in higher plants from 1949 onwards in the work of Hewitt and Jones (1950, 1951) [9]) [10]) and Agarwala (1950 unpublished).

TABLE II

Results Obtained by Comparison of A.R. and Recrystallised A.R. Grade Salts with Fully Purified Materials

Salt Replacement	Condition	Yield of Mycelium Dry wt. mg (Mean of (3 replicates)	Calculated Mo Value	
			Introduced to Culture μg	p.p.m. in Salt
KNO_3 Alone	A.R. Batch (1)	186	0.0007	0.0028
	2X Recrystallised A.R.			
	Batch (1)	74	0.0002	0.0008
	Batch (2)	335	0.0012	0.0047
$MgSO_4$	A.R. Batch (1)	897	0.0075	0.15
	A.R. Batch (3)	437	0.0026	0.052
Alone	3X Recrystallised A.R.			
	Batch (1)	210	0.00082	0.0167
	Batch (2)	140	0.00034	0.0096
	Batch (3)	455	0.0027	0.054
NaH_2PO_4 Alone	A.R. Batch (1)	154	0.0005	0.004
	2X Recrystallised A.R.			
	Batch (1)	356	0.0017	0.014
	Batch (2)	732	0.00375	0.03
All three together	A.R. not purified	940	> 0.01	> 0.02 in aggregate
	A.R. and Purified by CuS pptn.	90	0.0003	0.0006 in aggregate
	Recrystallised A.R. and purified by CuS pptn.			
	Batch (1)	35.5	NIL	—
	Batch (2)	36.0	NIL	—

The method, which depends on the co-precipitation of molybdenum and copper sulphides, was carried out as follows. Concentrated (5× normal) stock solutions were prepared for glucose plus sodium phosphate and potassium nitrate plus magnesium sulphate. For each litre was added 10 ml of 19.64% $CuSO_4 5H_2O$ and stirred. This produced a copper concentration of 500 p.p.m. The pH was reduced to 2.5 with HCl. H_2S, from a freshly prepared Kipp's generator with a splash trap and filter of cotton wool, was passed rapidly for 30 minutes. The heavy precipitate was left to settle overnight and removed by filtration through a No. 4 (2 l capacity) pyrex glass sinter under reduced pressure. The clear solution was tested with more H_2S and treated again if a precipitate formed. When all the copper was precipitated the filtrate was heated on a hot plate to

drive off H_2S, and to precipitate colloidal copper sulphide and sulphur which were removed by a final filtration. In some instances, especially in the presence of the sugar, precipitation was incomplete and colloidal material persisted after repeated filtration. The addition of a few drops of 20% HCl and heating after passing more H_2S was effective in completing precipitation. It was convenient and quite satisfactory to apply the procedure to solutions containing all four basal constituents.

The whole procedure was repeated a second time for the most critical tests and in many standard assay culture media. The effects of single or double precipitation are shown in table VII together with data relating to other methods. The sinter filter was rinsed by distilled water only between filtration of the respective stocks at any one time. When a batch had been completed the copper sulphide was removed by dissolving it in warm 1 : 5 nitric-hydrochloric acid mixture and copious rinsing with glass distilled water.

The removal of the precipitate from the filter was considered one of the weakest (or most vulnerable) points of the technique. Comparison of yields obtained by standard purification and after adding "washings" from the cleaned filter ready for use again or using No. 42 paper filters in place of the glass sinter gave the following results in table III.

TABLE III

Molybdenum Contamination Arising during Filtration	
Treatment	Mean Yield
NIL (Double CuS pptn.)	18 mg
0.00015 μg Mo	32 mg
0.0003 μg Mo	59 mg
NIL plus 40 ml "washings" from "cleaned" sinter	35 mg
Effect of filter paper; Solution filtered 5 times through layer of 5 5.5 cm papers	179 mg equivalent to .0005 μg Mo per sheet i.e. .000021 μg Mo per sq. cm
Molybdenum extracted from filter paper by hot water 20 ml extract equivalent to a single 11 cm sheet	306 = 0.002 μg Mo i.e. .000021 μg Mo per sq. cm

Obviously the importance of scrupulous care at this stage cannot be overemphasised. The amount of molybdenum supplied by about

1–3 ml of drainage water left in the cleaned sinter would only represent about .00001 μg Mo.

The micronutrient salts, except iron, were recrystallised twice from glass distilled water. Ferric chloride was prepared by a procedure designed to eliminate several micro elements including copper, zinc, manganese, gallium and molybdenum. The procedure was based on that originally developed by Hewitt for work with higher plants and used in the work of Hewitt and Jones (1949, 1950, 1951) [8] [9] [10]. It was as follows:

500 g A.R. $FeSO_4(NH_4)_2SO_47H_2O$ were twice recrystallised from glass distilled water with the addition of alcohol for efficient recovery. The washed crystals were redissolved in the minimal volume of water and the pH adjusted to 2.8 with pure hydrochloric acid. 0.75 g hydroxylamine sulphate was added to reduce any ferric iron and the solution extracted several times with 0.1% 8-hydroxyquinoline (oxine) in chloroform. This procedure was intended to assist in removal of molybdenum, gallium and certain other microelements. After removal of residual oxine with chloroform A.R. concentrated hydrochloric acid was added to produce an acidity of 6N. Any ammonium chloride that precipitated was removed and the solution saturated with excess of distilled ether and extracted several times. Gallium and molybdenum were assumed to be concentrated into the ether phase which was discarded, cf. Sandell 1944) [25].

The ferrous iron concentration in the solution was determined and the theoretical equivalent (less about 1 per cent) of hydrogen peroxide was added to convert ferrous to ferric iron. The hydrogen peroxide was first purified by distillation under reduced pressure in an all glass apparatus followed by extraction with oxine in chloroform at pH 2.8. The ferric chloride solution was evaporated practically to crystallising point and then diluted with redistilled (20 per cent) hydrochloric acid to 6N concentration. This solution was saturated with excess redistilled ether and extracted three times with 200 ml portions of ether. The ether phase which now contained the ferric chloride was retained. The aqueous acid phase containing copper, zinc, manganese, cobalt and other heavy metals was rejected. The ether fractions were bulked and washed twice with 200 ml portions of redistilled 6N hydrochloric acid saturated with ether. The ferric chloride was then extracted three times with 200 ml portions of glass distilled water. This solution was stored in a pyrex glass bottle and the iron content determined. (The usual concentration was about 40–50 g $Fe^{\cdots}$/l). Ferric citrate (5.6 mg $Fe^{\cdots}$/ml) was prepared from this stock and from purified citric acid, by evaporation together with frequent stirring on a water bath fitted with porcelain rings until all hydrochloric acid was expelled and a viscous or partly solid mass remained. This was redissolved in 1 *l* glass distilled water and stored in the dark. The citric acid was purified by recrystallisation followed by treatment with copper and hydrogen sulphide as for the macronutrient stocks.

Inoculation and Incubation.

Cultures were inoculated, after sterilisation for 15 minutes at 110°C. (15 lbs. p.s.i.), from a spore suspension in sterile distilled water. Normally 0.1 ml of the suspension delivered from a 1 ml pipette was introduced into each culture.

Two aspects of inoculation were tested, namely the effects on yield of spore numbers and of molybdenum status of the spore culture. An attempt was made to estimate the probable limits of molybdenum introduced by the spore inoculation. It will be seen from data given for the standard growth curves in table V that sporulation can rarely occur when less than 0.005 μg Mo per 50 ml is present. Hence all spores must be prepared from agar slopes or controlled cultures containing at least 0.005 μg Mo per 50 ml if nitrate nitrogen is used. The problem of contamination of the spore inoculum by contact with the agar or culture fluid whilst collecting the spores is a mechanical one and only care can minimise it. The problem of molybdenum content of the spores is physiological. The results in the first part of table IV must naturally be the sum of both effects: one kept to the minimum permitted by manipulation. In the preparation of all routine spore suspensions a platinum loop was used to transfer the spores and mechanical contamination was possibly greater in some instances than others. In the second group of results shown in table IV the spores were transferred by detaching them by suction in a stream of air. A narrow tube drawn to a jet at one end and directed at the other end over sterile distilled water in a small wide tube connected to a vacuum pump was "scanned" over the surface of freely sporing culture after partial drying of the mycelium. The method was similar to that given by McCallan and Wilcoxon (1936) [12]. Spore

TABLE IV

Examples of Effect of Spore Concentration and Source of Spores on Yield of Mycelium

Spore Count per ml	No. of Spores per culture	Origin of Spore	Mean Yields mg	
			"NIL"	0.01 μg Mo added
5,000,000	500,000	Agar Slope	65.5	754
1,500,000	150,000	,,	44.0	1041
1,030,000	120,000	,,	43.5	977
1,000,000	100,000	,,	55.0	823
760,000	90,000	,,	44.0	875
200,000	20,000	,,	25.5	823
		Controlled Cultures grown with added Mo		
1,500,000	150,000	.005 μg	128	—
1,500,000	150,000	.01 μg	155	—
1,500,000	150,000	.1 μg	202	—
1,500,000	150,000	.5 μg	41.5	—
Fragment of mucous mycelium from "Nil" culture:			107	

counts were made on the suspension before using it for inoculation. There was some suggestion that yields of cultures obtained in the absence of extra molybdenum from such spore suspensions increased up to a point with molybdenum level used to develop the sporing mycelia, and then declined possibly due to a toxic influence of excess molybdenum.

The time of incubation was varied to find the most convenient period. $5\frac{1}{2}$ days was finally adopted as the most suitable to give a satisfactory range from "low" to "high" together with high yields for the top region of the curve, and free sporulation. When necessary cultures were left for 6 days without noticeable detriment.

It was found difficult to maintain the incubator at a closely determined temperature indefinitely and it was more convenient to incubate at the temperature prevailing providing this did not exceed 31° or fall below 25°C. In practice the great majority of the work was carried out at 26°C. Temperarures did not normally vary more than 0.5°C during incubation. It was noticed (without means of quantitative proof) that richly sporulated cultures often had a slightly lower dry weight than those less profusely sporulated at the same molybdenum level. Data of Löhnis (1944) [11] show a similar phenomenon. A possible explanation is that respiration rate may rise when sporulation is profuse and that under such circumstances loss of dry weight may exceed further glucose assimilation. For this possible reason it was considered desirable to incubate at a relatively low temperature (25°–28°C) rather than at the higher temperatures (30°–32°C) used by Steinberg (1936, 1937) [28] [29] and Mulder (1948*a*, *b*) [16] [17]. Table V shows that the lower temperature produced greater yields with adequate molybdenum.

It was also observed that sporulation seemed to have become less profuse in 0.01 μg cultures with time and that 0.02–0.05 μg Mo was needed to obtain profuse sporulation in the latter part of the work. The yields recorded for low levels of molybdenum even in early work, and the shape of the growth curve would appear to exclude the suggestion that the change was due to the extraction of actual molybdenum reserves over a period of years but no alternative explanation can be offered at present.

Harvesting mycelia.

The yields were determined as the dry weights of mycelia grown in duplicate or triplicate cultures and dried at 75°C for 18 hours. The dry weight of unused glucose and nutrient salts was considered a likely source of error in these determinations especially when low yields were concerned, as most of the glucose was still present in the culture fluid. The mycelial felts were therefore washed thoroughly before drying.

The procedure used consisted in first dipping the "felt" in alcohol if sporing, to kill spores and lessen contamination of the atmosphere by high concentrations of spores. It was found that all felts even when completely mucous had sufficient cohesion to allow of them being shaken out of the culture flask in one piece into a glass dish. The felt was then thoroughly washed in water and moved "to and fro" with a glass rod. The water was continually replaced and removed from the dish by suitably adjusted inlet and outlet tubes, the latter attached to a filter pump. A few mycelia were tested for absence or presence of residual reducing sugar by Fehling's solution used on a small volume of water after agitation in contact with the washed mycelium. A minimum washing time of 3 minutes was necessary. The "felts" were then blotted between several sheets of clean absorbent paper and dried on weighed watch glasses each kept inside a pair of petri dishes during manipulation.

The Growth Curve.

The molybdenum values were generally determined by reference to a growth calibration curve prepared each time from a series of duplicate cultures given known concentrations of molybdenum. Table V shows the values obtained for growth calibration curves prepared during the later stages of this work when overall technique was at a high level and during the early stages before maximum efficiency was attained. The mean values obtained from several growth curves are given for incubation at different temperatures. Examples of these growth curves and the 5% fiducial limits for a mean curve based on 10 determinations recorded during 1949 are shown in figures 1–3 *).

Fig. 4 shows two curves (early and later work) plotted with arithmetical molybdenum scale to indicate the probable value of "nil" levels from the intercepts. These are apparently 0.0004 μg and 0.0001 μg respectively. A typical growth series is illustrated in photograph No. 2.

It was thought of interest to find to what extent the unabsorbed nitrate reflected severity of molybdenum deficiency. The nitrate values were determined on a number of occasions and the figures in tableV are the means of several determinations.The typical appearance of cultures is also given as visual appearance was a useful guide in the general evaluation of results. Abnormality in sporulation or

*) The data for fig. 3 were calculated from 10 results in 1949 by the Rothamsted Statistical Department and the original curve was constructed by Dr G. M. Jolly whose assistance is gratefully acknowledged.

TABLE V

Data for Representative Growth Curves and Residual Nitrate in Culture Fluid								
			Growth Curves			Mean Standard Growth Curves		
Molybdenum level μg	Mean Residual NO_3 level μg/ml	Typical Appearance	(Early Work) mean of 2 cultures	(Later Work) mean of 2 cultures		(*a*) 25–26°C Means of 24	(*b*) 31°C Means of 6	(*c*) 33°C Means of 2
				(*a*)	(*b*)			
"nil"	325	Thin mucous mycelium submerged in culture fluid	88	29	23.5	42.0	41.3	32
0.00015	285	— do —	97	41	46	74.2	86.0	86
0.0003	213	semi-translucent mucous and partly surface growth	155	75	57	91.9	145	201
0.0006	170	— do — slightly more opaque	206	140	135	159.0	238	239
0.00125	130	opaque with some white patches, predominantly surface	373	215	196	263.0	315	328
0.0025	7.5	thick white mycelium partly surface and aerial growth	452	271	402	446.1	390	422
0.005	5	yellow pigment lower surface. Patchy sporulation. Mycelium tough, leathery and corrugated	852	576	700	784.5	694	768
0.01	—	— do — and abundant sporulation	894	819	1057	902.5	750	695
0.02		— do —	839	—	—	945.3 (mean of 6)	—	—

Maximum values recorded for "nil", 96 mg; for .01 μg, 1057 mg (25°C).
Minimum values recorded for "nil", 15 mg; for .01 μg, 665 mg (31°C).
53% of all "nil" values were below 40 mg.
Mean yield ratio % of "nil" to .02 μg. 22.7 : 1 or 4.42%.
Maximum ratio ,, 44.7 : 1 or 2.23% (mean of 2 cultures).
Minimum ratio ,, 9.16 : 1 or 10.9% (mean of 2 cultures).
Low temperature curve more sensitive at high levels of Mo.
High temperature curve more sensitive at low levels of Mo.

mycelial distribution which occasionally occured, was taken to indicate possible toxicity or local contamination where these could be reasonably anticipated. It was found that by removal of the residual culture fluid after 5 days followed by further incubation, profuse sporulation was induced at the 0.005 μg level. The apparent

reduction in sporulation at .01 μg Mo levels during the period of experiment has already been noted.

Yields obtained showed that for "nil" additions of molybdenum, growth was normally reduced to about 5% or less of the full yield.

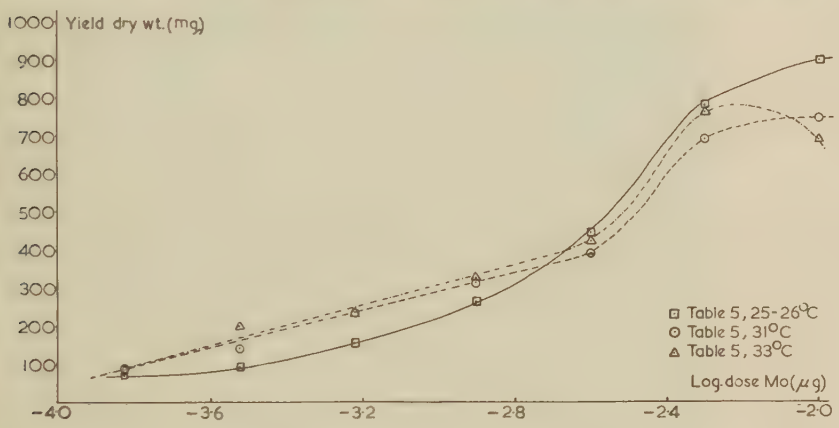

Fig. 1. Mean growth curves for *A. niger* at various temperatures with increasing molybdenum status.

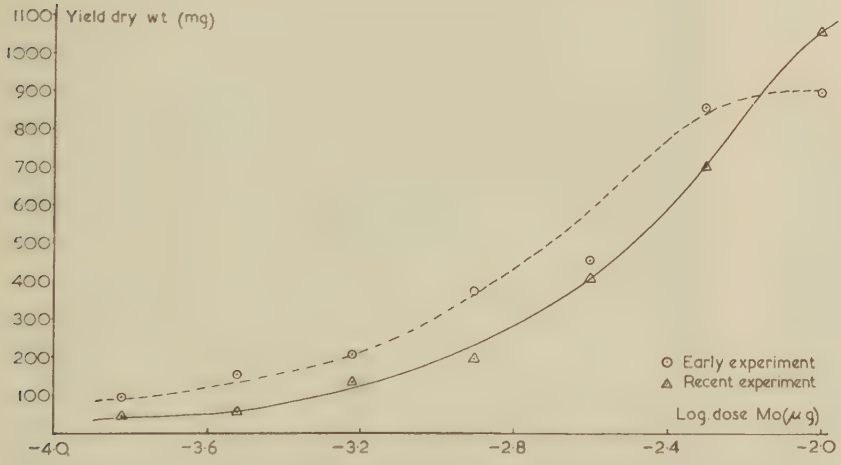

Fig. 2. Growth Curves obtained during early and later stages of the work (table V).

It was possible consistently to detect 0.00015 μg Mo (with few exceptions due to faulty technique) and the interval readily allowed the interpolation of as little as 0.00005 μg Mo.

When plotted on arithmetical co-ordinates, the curve for growth at 25–26°C, was practically linear between very low values and 0.005 μg Mo (fig. 4). Above these values sensitivity was greatly reduced and there was no response beyond 0.02 μg and often not beyond 0.01 μg Mo. For practical purposes of assay 0.01 μg was considered

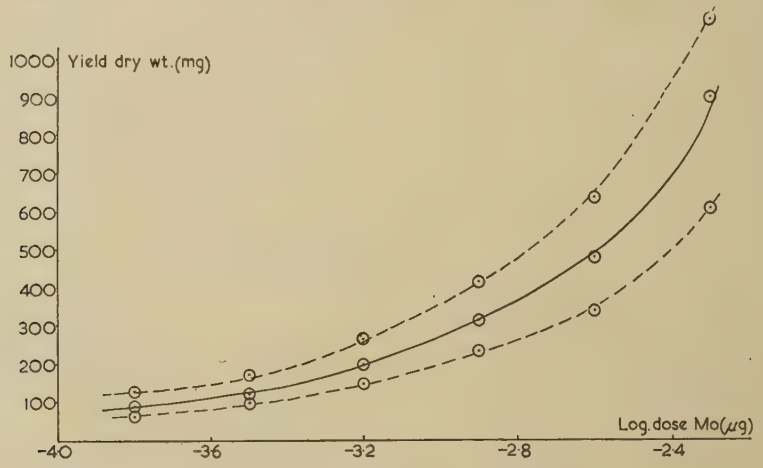

Fig. 3. 5 per cent fiducial limits for mean of 10 determinations between 0.00015 and 0.005 μg Mo (courtesy of Rothamsted Experimental Station).

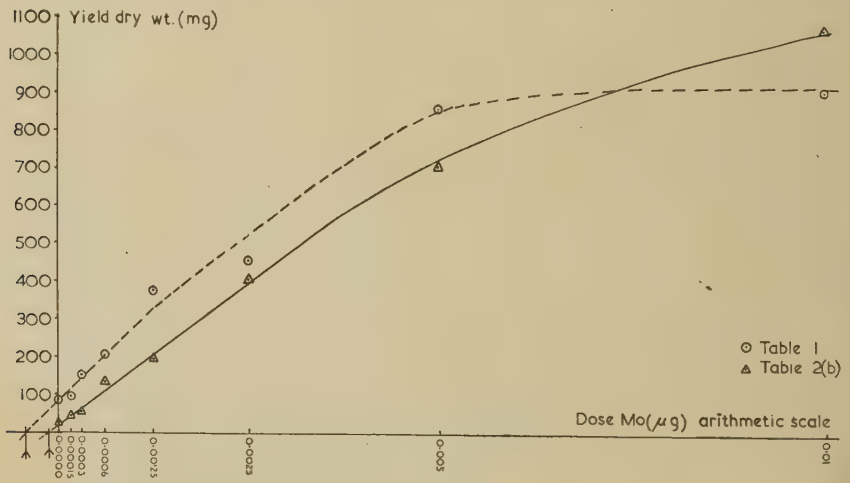

Fig. 4. Data of fig. 2 shown with arithmetical coordinates and intercepts for estimation of probable total molybdenum contamination. ⊙ Early experiment intercept = 0.0004 μg Mo. △ Recent experiment intercept = 0.0001 μg Mo.

Plate 1

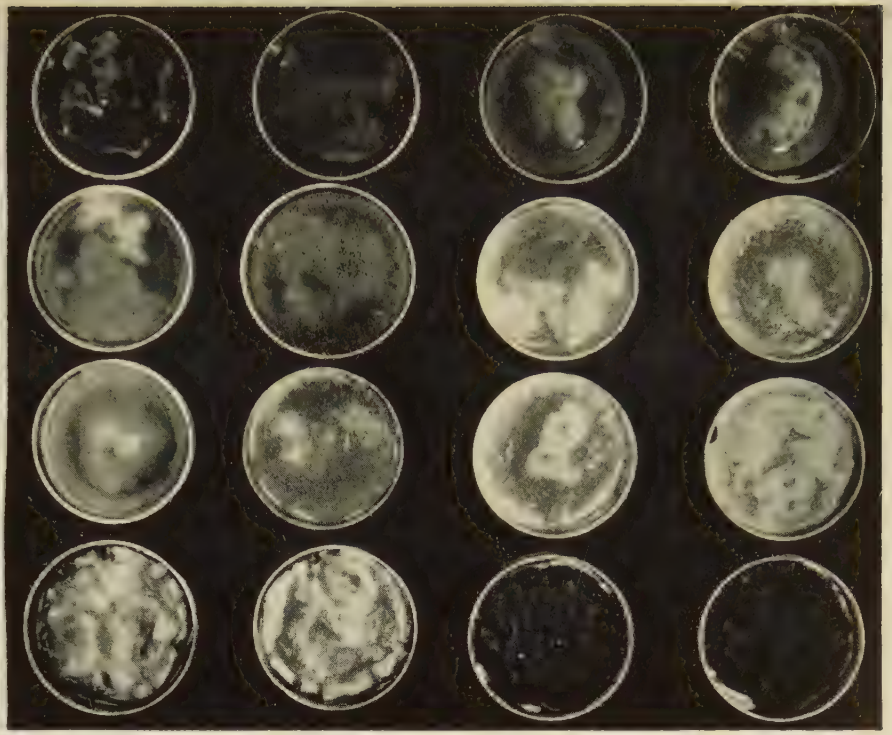

Plate 2

Plate 1. Effect of cotton wool. Above, with cotton wool plugs. Below, with pyrex glass beaker.

Plate 2. Growth series at 25° C. In pairs left to right: top line, nil, 0.00015 μg; 2nd line, 0.0003 μg, 0.0006 μg; 3rd line, 0.00125 μg, 0.0025 μg; bottom line, 0.005 μg, 0.01 μg.

Plate 3a

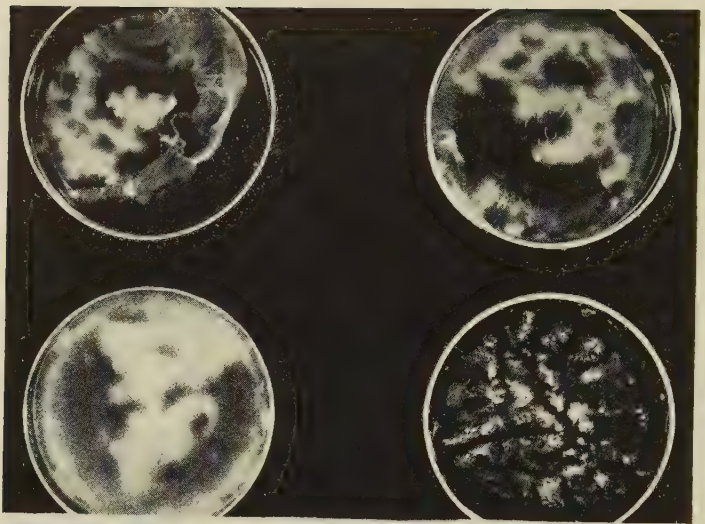

Plate 3b

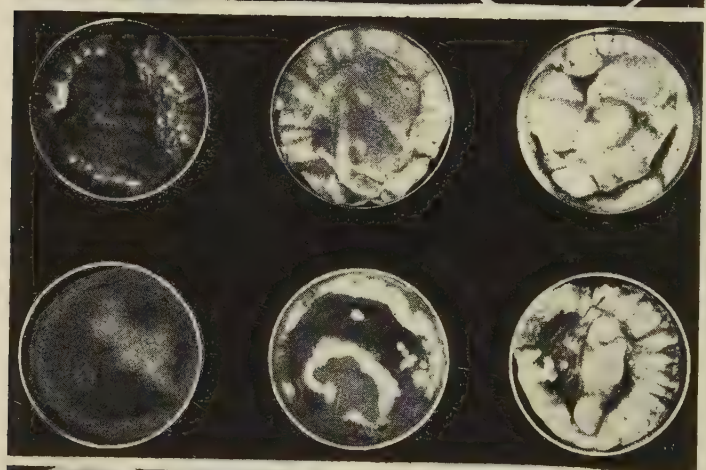

Plate 4

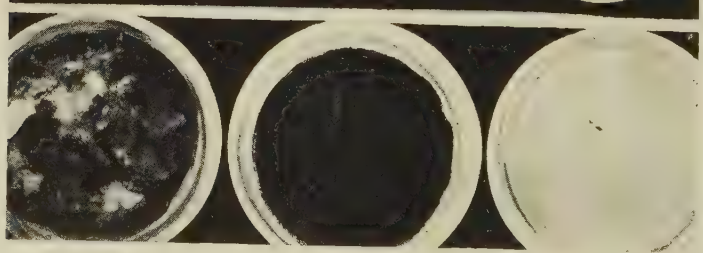

Plate 3a. Removal of molybdenum by copper sulphide. Top left, 0.1 μg Mo, added before purification; bottom left, 1.0 μg Mo added before purification; top right, blank (nil Mo added); bottom right, 0.01 μg Mo.

Plate 3b. Effects of copper and molybdenum concentrations on molybdenum removal by sulphide co-precipitation. Top line (l.to r.) 4 μg Mo added, Cu at 25, 500 and 1000 ppm; bottom line (l.to r.) Cu at 500 ppm, 0.25, 1.0 and 4.0 μg Mo added.

Plate 4. Effect of using unpurified salts or method of sulphide co-precipitation. Left, no Mo, AR salts recrystallised but not purified further; centre, CuS precipitated by ammonium sulphide; right, CuS precipitated by H_2S.

the upper limit of determination and if possible test samples were selected to contain between 0.0001 and 0.005 μg Mo for best results.

Inspection of the mean curves in fig. 2 obtained for growth at 26° and 31°C. suggested that the higher temperature reduced yields in the presence of adequate molybdenum but slightly increased rate of growth at the lowest levels above the "nil". The probits plotted against yields indicated that the curve for 26°C was practically linear but for 31°C it was slightly concave with apparently a break at or just above 0.0025 μg *). This level of molybdenum corresponded roughly with the physiological transition between an entirely non sporulated mycelium and patchy sporulation. This was usual at 0.005 μg and only rarely observed in cultures where yield indicated a probable molybdenum level of 0.003–0.004 μg by interpolation. On a logarithmic scale of molybdenum levels between 0.00015 and 0.02 μg the growth curve at 26°C was practically sigmoid. At 31°C it was similar but less regular, and showed reduced sensitivity.

Statistical treatment of this work and the application of a two point assay procedure will be dealt with on a separate occasion. For the purposes described here simple interpolation of duplicate or triplicate determinations on a standard growth curve prepared at the same time were used to estimate the molybdenum level. The appearance of the mean curve at 26°C suggested that this temperature was more suitable for the purposes of the work.

The Efficiency of the Standard Purification Method.

The efficiency of a purification method is defined here as the percentage removal of a known concentration of molybdenum from an initially highly purified medium. To a basal medium already purified twice as described above were added molybdenum and copper at various concentrations. The yields were determined and the residual molybdenum concentration estimated. The efficiency of removal, E was then determined as $E \frac{M - m}{M} \times 100$, where M and m were the added and residual molybdenum concentrations respectively. The results of typical experiments are shown in table VI and in photograph no. 3.

The results in table VI indicate that removal of molybdenum is

*) The assistance of Mr. A. Stringer, B.Sc. of Long Ashton is gratefully acknowledged in this connection.

TABLE VI

Efficiency of Molybdenum Removal by Standard Purification Method				
Mo added* μg (per culture)	Cu Concentration	Yield mg	Mean μg Mo remaining	% Removal
		I		
0.02	500 p.p.m.	39.0 69.6 45.4	0.00003 approx.	99.85
0.10	,,	60.0 82.7 90.2	0.000094	99.91
0.20	,,	235.9 263.8 337.4	0.0010	99.50
0.20	5000 p.p.m.	96.2 81.3 83.2	0.00013	99.93
		II		
0.25	25	231 288	0.00096 0.0013 .0011	99.56
	100	86 105	0.00016 0.00018 .00014	99.94
	500	64 79	0.00003 0.00006 .000045	> 99.98
	1000	47 88	Nil 0.0001 Nil	> 99.99
1.0	25	346 469	0.0017 0.00255 0.0021	99.79
	100	240 185	0.0010 0.0007 .00085	99.91
	500	172 153	0.00057 0.00045 .00051	99.95
	1000	94 120	0.00012 0.00027 .00018	99.98
4.0	25	770 927	0.0045 0.01 .0073	99.81
	100	584 718	0.00325 0.00415 .0037	99.91
	500	365 434	0.0018 0.00225 .0020	99.95
	1000	247 292	0.00105 0.00135 0.0012	99.97

*) The molybdenum concentration actually treated with copper sulphide was 5 times that shown as the purification was carried out on the concentrated medium before dilution. Thus the estimated removal was almost certainly not less than the value given. This was because comparison with the standard series was made at the low end of the curve and the yields obtained were often not much greater than the irreducible minimum association with "nil" values.

progressively improved by increase in the copper concentration and is less efficient at high than at relatively low levels of molybdenum. The maximum efficiency that could be determined attained 99.99% and as much as 1 μg Mo could be removed with an efficiency of 99.98%.

The marked drop in efficiency in the presence of 4 μg Mo suggests that available surface of copper sulphide was practically saturated as the difference between efficiencies in the presence of 0.25 and 1 μg were comparatively much smaller.

In the presence of much lower concentrations, however, of the order of .02 or .002 .005 μg shown in tables VI and VII there appeared to be a slight drop in efficiency. The difficulties of estimating accurately the residual molybdenum concentration after purification meant that a cautious estimation of per cent efficiency tended to be low. On the other hand the possibility that traces of molybdenum were less efficiently collected than intermediate concentrations, especially when low concentrations of copper were used, cannot be excluded at present. The use of a radio-active isotope would assist in deciding this point. However, the addition of molybdenum at 10 μg per 200 ml concentrated solution as stated by Nicholas and Fielding (1950) [20] is greatly in excess of the critical concentration required to produce the possible effect noted here. The experience obtained in this work would suggest that such an added concentration could reduce rather than enhance the efficiency of purification. That molybdenum sulphide alone in the presence of iodine (discussed below) is not readily removed without a collector is further evidence for the contention that addition of appreciable molybdenum is undesirable. The standard procedure (500 p.p.m. Cu) should apparently be adequate to handle levels of molybdenum introduced by A.R. grade salts and distilled water (tables II and IX).

The Efficiency of Other Methods of Purification.

Prior to the work of Hewitt and Jones (1947) [7] methods used in work intended to demonstrate molybdenum deficiency did not eliminate this element with any great efficiency. Successfull results probably depended rather on the accidental absence of molybdenum and a generally high standard of technique. Methods formerly used included the phosphate and alkaline earth adsorption techniques of Steinberg (1935) [27] and Stout and Arnon

(1939) [31]) as applied by Arnon and Stout (1939) [1]), or combined with recrystallisation as in the work of Piper (1940) [22]). The first attempt specifically to eliminate molybdenum was made by Hewitt and Jones (1947) (1949) [7]) [8]) by use of co-precipitation of molybdenum with 8-hydroxyquinoline and iron. The method was shown to be satisfactory and efficient for deficiency culture experiments and was based on techniques devised by Scott and Mitchell (1943) [26]) and Mitchell and Scott 1948 [14]) to concentrate microelements quantitatively from soil and plant extracts. Later Nicholas and Fielding (1948) [19]) also employed 8-hydroxyquinoline for this purpose. Other simple methods for the removal of molybdenum considered here include co-precipitation with sulphides other than of copper. A method of especial interest in the treatment of glucose (or non-ionised materials and water) is that of passage through synthetic resins. The use of these materials is discussed in the next section. Table VII shows the effects of using precipitation procedures with solutions made up from A.R. grade salts alone, or after the addition of a known concentration of molybdenum to an already highly purified medium. The value of recrystallisation (table II) has already been discussed. The procedures employed do not justify description here in detail. The phosphate adsorption and 8-hydroxyquinoline and iron co-precipitation methods were carried out in a similar way to descriptions already published *loc. cit.* The sulphide co-precipitations were analogous in procedure to the standard method. In each, 500 p.p.m. of the metal or mixture of metals were used (unless shown otherwise) and a single precipitation was carried out.

The general order of efficiency for different sulphides is seen to be consistently high. The difference in yield obtained in experiments separated by long intervals reflects partly improved efficiency with time, partly the initial molybdenum concentration and partly uncontrolled variation in efficiency and in yield for a particular growth series.

The sulphides fell into roughly 3 groups with regard to efficiency of removal as follows:

Good: mixed sulphides; CuS, PbS, As_2S_3 alone.

Fair: AgS, HgS.

Poor: CdS or sulphides precipitated by $(NH_4)_2S$ solution.

The low efficiency of cadmium sulphide used *alone* is interesting.

TABLE VII

Comparison of Several Purification Methods

Initial Mo level μg	Method	Yield	Estimated residual Mo μg	Mean Estimated Efficiency %
0.0053 approx.	Precipitation of Alkaline-earth phosphate	596	0.0027	37.6
		705	0.0039	
0.0053 approx.	Co-precipitation by copper sulphide using $(NH_4)_2S$	518	0.0024	50.0
		604	0.0028	
0.0053 approx.	Co-precipitation with 8-hydroxyquinoline	240	0.00070	88.6
		175	0.00059	
0.005	— do —	123	0.00024	92.4
		192	0.00055	
		146	0.00030	94.3
		148	0.00030	
		132	0.00025	
0.02	— do —	167	0.00043	
		256	0.00070	96.3
		346	0.0011	
0.0053 approx.	Co-precipitation with 8-hydroxyquinoline followed by CuS	224	0.00060	92.8
		92	0.00015	
0.02	— do —	203	0.00057	
		187	0.00050	97.85
		100	0.00022	
0.005	Co-precipitation with CuS followed by 8-hydroxy-quinoline	48	0.00010	
		85	0.00015	97.4
		62	0.00013	
0.0053 approx.	— do —	123	0.00024	
		192	0.00055	92.7
0.02	— do —	95	0.00018	
		159	0.00040	98.3
		181	0.00045	
	Single co-precipitation by sulphides			
0.0053 approx.	CuS 500 p.p.m. Cu	93	0.00015	98.2
		45	0.00005	
	CuS + PbS 500 p.p.m. each metal	77	0.00010	98.1
		82	0.00012	
		49	all "nil"	> 99.9
		33		
		39		

This effect was frequently confirmed in early work where, although technique was not as good as the standard finally achieved, yields of "nil" cultures purified with cadmium sulphide were 85% of full

TABEL VII (continued)

Initial Mo level μg	Method	Yield	Estimated residual Mo μg	Mean Estimated Efficiency %
0.02	CuS 500 p.p.m. Cu	203	0.0005	
		193	0.00047	97.75
		177	0.00042	
	CdS ,, Cd	410	0.0026	
		570	0.0045	84.0
		399	0.0025	
	PbS ,, Pb	335	0.00125	
		146	0.0003	95.8
		323	0.00123	
	CuS + CdS 500 p.p.m. each metal	25	"nil"	> 99.9
		54		
		65		
	CuS + PbS 500 p.p.m. each metal	49	"nil"	99.94
		45	"nil"	
		185	0.00035	
1.0	SnS_2 500 p.p.m. Sn	No precipitate obtained		
	As_2S_3 500 p.p.m. As	69	0.00045	99.95
	CuS ,, Cu	41	0.00015	99.96
		87	0.00060	
	CdS ,, Cd	649	0.005	99.5
		649		
	CuS + CdS 250 p.p.m. each metal	21	"nil"	> 99.99
		35		
	PbS 500 p.p.m. Pb	100	0.00067	99.93
		103	0.0007	
	CuS + Pbs 250 p.p.m. each metal	112	0.00080	99.94
		77	0.00050	
	AgS 500 p.p.m. Ag	177	0.00125	99. 88
		166	0.00120	
	HgS 500 p.p.m. Hg	256	0.00154	99.83
		310	0.00190	
0.0027 approx.	CuS twice precipitated	21	"nil"	99.95
		33		
	CuS once precipitated	46	—	96.3
		52		
	PbS + CdS ,,	49	—	99.9
	(500 p.p.m. each)	33	—	
		39	—	

yield whilst with copper and other sulphides yields were reduced to 10–20% of full yield and often much less. No obvious explanation can be suggested at present but a study of the surface of the cad-

mium sulphides in relation to ionic spacing, etc., might afford useful insight into the problem.

Cadmium (alone or with other metals) appeared to exert a toxic effect on cultures given the full level of molybdenum as sporulation was usually markedly inhibited or lacked pigment. The dry weights when molybdenum was given, however, were not greatly reduced after using cadmium or any other sulphide method and sporulation in all other methods was normal. The data are not given here but in all instances yields ranged between 750 and 1000 mg in the presence of 0.01 μg Mo. These results indicate that here toxic effects were not significant in the presence of adequate molybdenum. Some of the results are illustrated in photograph no. 4.

Feigl (1949) [3] pp. 163-164 and 467–468 pointed out that the precipitation of molybdenum sulphide is greatly improved by the presence of iodine. The precipitate is coagulated and the operation rendered nearly quantitative at low concentrations of molybdenum. The iodine converts part of the hydrogen sulphide to sulphur which coagulates the molybdenum sulphide. This modification was used by Nicholas and Fielding (1951) [21]).

The effect of using iodine in the presence of added molybdenum and hydrogen sulphide was tested by us in the presence and absence of copper.

TABLE VIII

Effect of Using Iodine in Sulphide Precipitation

Treatment	Mo level *)	Mean Yield mg †)	
		— Mo	+ Mo
CuS alone	1.25 μg	26.5	784
CuS + 10 p.p.m. I_2		60.0	723
10 p.p.m. I_2 only		924	945
CuS alone	5 μg	39.5	769
CuS + 10 p.p.m. I_2		32.0	791
CuS + 100 p.p.m. I_2		31.5	876
10 p.p.m. I_2 only		688	814
100 p.p.m. I_2 only		963	777
CuS alone	20 μg	32	936
CuS + 10 p.p.m. I_2		69.5	815
10 p.p.m. I_2 only		752	603

*) Concentrations in solution treated were 5 × those finally present in diluted culture fluid.

†) The + Mo cultures were given 0.01 μg Mo after repurification to test for possible toxicity effects.

The iodine was added as an aqueous solution in potassium iodide. Copper when present was at a concentration of 500 p.p.m. The total iodide concentration varied between 4 p.p.m. and 20 p.p.m. in diluted nutrient used to grow the fungus. The results are given in table VIII. The addition of iodine did not appear to improve the efficiency in the presence of copper and was useless alone. The iodide concentration did not appear to be toxic. The method may, however, be of value in the purification of certain types of solution. Thus in biochemical work it may be required to purify a phosphate buffer with or without glucose. At the pH of 6–8 incomplete precipitation occurs and the colloidal material cannot be easily removed. Addition of iodine greatly improves coagulation under these circumstances and filtration is satisfactory.

Removal of molybdenum by ion-exchange resins.

The ion-exchange resins may be used with advantage to remove traces of metal impurities from dilute solution. They cannot readily be applied to the purification of the reagent salts because these are ionised. Glucose and water, however, may be treated in this manner. Examples of the results obtained by passing solutions of glucose, or water through some ion-exchange resins after addition of known amounts of molybdenum are shown in table IX.

The resin treatment consisted in passing through a column 2.2 cm diam. × 35 cm length a 50% solution of glucose to which 1 μg Mo per l had been added. The resins used were the IMAC C11 product of Industrieele Activit Maatschappij Amsterdam and Permutit Deacidite E and Biodeminralit. The first 30 ml of perfusate was rejected. This was equivalent to considerably more than the aqueous phase displaced from the column. A suitable aliquot was then used for each culture.

There was a tendency toward more efficient removal at a lower pH value. Deacidite F was less efficient than anticipated. This resin is designed for dealing with weakly dissociated ions. The result was the more remarkable as Deacidite E appeared more efficient. The explanation suggested here is, that as the tests were made with solutions of sodium molybdate, hydrolysis occurred at the resin surface and the local high pH tended to cause desorption of the anion. Under these circumstances the more basic Deacidite E would probably be the more efficient of the two. The Biodeminralit would be expected to show intermediate behaviour as was found. Trials with molybdic acid are required to test this point.

TABLE IX

Removal of Molybdenum by Ion Exchange Resins

Material	Initial Mo concentration (μg per culture)	Yield mg	Estimated Mean Mo concentration (μg per culture)	% Removal
Glucose passed thro' resins	0.0055 (as Na_2MoO_4)	556 513 605	0.0028	51
a. Permutit Deacidite E *b.* Industrieele Activit IMAC C11		46 55 58	0.0001	98.25
2× Glass distilled water	NIL			
a. Permutit Deacidite E	Test on initial leachate 10 ml each	270 428	0.0014 (.00014 p.p.m.)	—
b. Industrieele Activit IMAC C11		79 87	0.0001 (.00001 p.p.m.)	—
a. Permutit Deacidite E	0.04 *) (as Na_2MoO_4)			
	at pH 8	42, 49, 83	0.00005	99.9
	pH 6	28, 62, 66	0.00005	99.9
	pH 4	34, 59, 75	0.00005	99.9
	pH 2	43, 48	"nil"	> 99.95
b. Permutit Deacidite E	0.04			
	at pH 8	133, 213	0.00044	98.90
	pH 6	149, 173, 272	0.00055	98.63
	pH 4	79, 90, 112	0.00022	99.45
	pH 2	52, 58, 70	0.00006	99.85
c. Permutit Biodemineralit (mixed bed resin)	0.04 at pH 8	134, 138	0.0003	99.25
	pH 6	46, 76, 136	0.00017	99.58
	pH 4	37, 41, 67	0.00003	99.93
	pH 2	26, 47, 26	"nil"	> 99.95
d. Activit IMAC C11	0.04			
	at pH 8	68, 85, 108	0.00017	99.58
	pH 6	50, 73, 75	0.0001	99.75
	pH 4	43, 45, 69	0.00005	99.90
	pH 2	30, 38, 38	"nil"	> 99.95

*) The actual concentration of molybdenum present in the water was 8 μg/l (0.008 p.p.m.). 5 ml of the effluent was used for each test and the maximum amount per culture was thus 0.04 μg. The relativily high concentration used and the small aliquot tended to minimise errors due to unsuspected contamination from the resins themselves, from the water suuply and during manipulation.

Mean residual Mo μg for all pH values;

a. 0.00006, *b.* 0.00025, *c.* 0.0001, *d.* 0.00006.

Mean residual Mo μg for all resins:

pH 8 0.00014; pH 6 0.00019; pH 4 0.000066; pH 2 0.00002.

PART TWO
THE ESTIMATION OF MOLYBDENUM IN MATERIALS USED IN CULTURE EXPERIMENTS AND IN PLANT MATERIAL

The needs of higher plants for molybdenum are sufficiently low to exclude the use of normal analytical methods for the detection of significant molybdenum impurities in culture materials such as sand, water and nutrient solutions. The *A. niger* technique described above has provided a useful approach to this problem and some of the results obtained in relation to the examination and purification of culture materials are given here.

The molybdenum content of deficient plants may also be too low for ready determination by chemical means since yields of dry material often do not exceed 2 g. This means that only 0.02–0.1 μg molybdenum may be present in all. In such circumstances a more reliable determination can often be made by assay with *A. niger*. Comparative data obtained in this way and by orthodox chemical analyses are given below.

Specificity.

Before proceeding to the results obtained in this part the question of specificity of response of molybdenum and certain other points bearing on the assay, need consideration.

It is known that vanadium can replace molybdenum under certain circumstances in the nutrition of *Azotobacter* and it was considered necessary to demonstrate the specificity of the method for molybdenum in the possible presence of elements such as vanadium or tungsten. Accordingly the effects of trying to replace molybdenum by each in turn were tested with the following results, shown also in photograph no. 5.

	Yield mg	Mean mg
"NIL"	38, 59	47.5
"NIL" + .005 μg V.	44, 35, 64	48.0
"NIL" + .005 μg W.	49, 60, 38	52.3
"NIL" + .01 μg Mo	900, 824	862

It was concluded that neither vanadium nor tungsten could effect such replacement and that the method was probably specific for molybdenum.

Two other aspects of the problem, however, had to be considered. Namely, whether traces of metal ions or of chelating agents that might remain from purification procedures would lead to false estimates of the true molybdenum status owing either to antagonism, or to immobilisation of molybdenum by the residual chelating agent. The latter possibility was

Plate 5

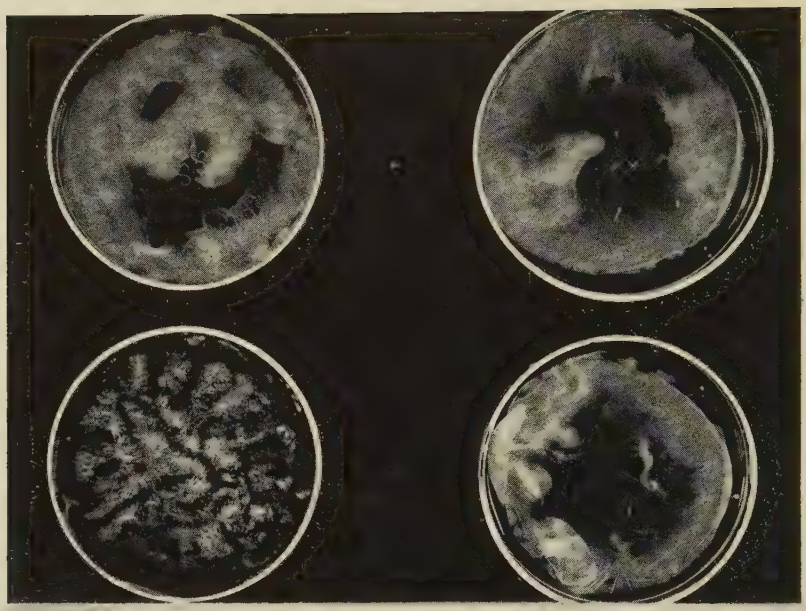

Plate 6

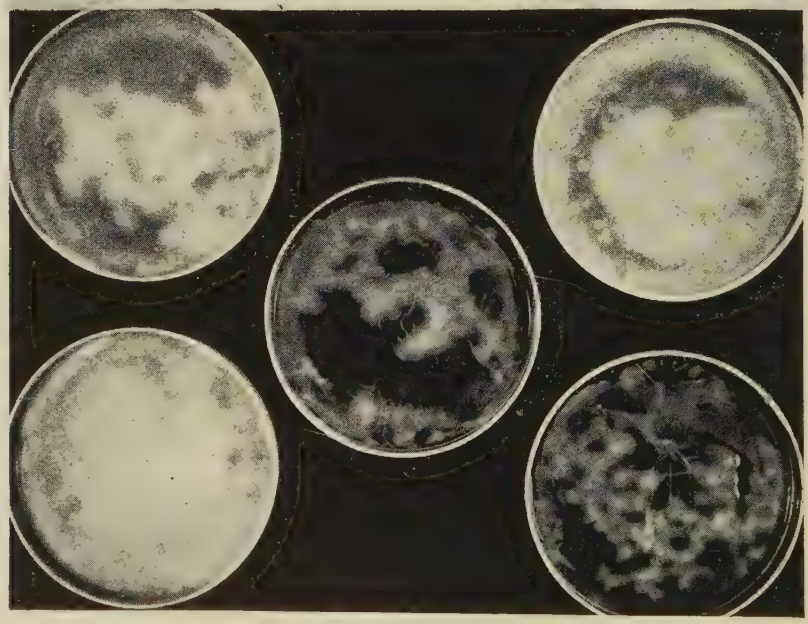

Plate 5. Effect of using vanadium and tungsten in absence of molybdenum. Top left, no Mo; top right, no Mo + 0.005 μg V; bottom left, 0.01 μg Mo; bottom right, no Mo + 0.005 μg W.

Plate 6. Examples of assay of Mo in water supply. Top left, 10 ml tap water; top right, 10 ml rain water (from tanks); bottom left, 30 ml demineralized rainwater; bottom right, 40 ml distilled water from tinned copper stills with silver condensers; centre, control "nil" with 2 × glass distilled water.

irrelevant when the sulphide method was employed but the possibility of a metal-molybdenum antagonism significant only at low concentrations of molybdenum was considered to be real. This conclusion was based on the observation that removal by dithizone of heavy metals from distilled water allowed the determination of a low level of molybdenum that previously has been masked. The data are given later for water assays. The effect of adding 2 μg silver or tin to the cultures was tested for molybdenum levels of 0.0006 and 0.01 μg. There was no effect on yields as compared with cultures grown in the absence of these metals at the same levels of molybdenum. The exact nature of the problem has not yet been elucidated and is still being studied.

The second problem concerned the finding that the assay of molybdenum in plant dried material resulted in a changed appearance in the mycelium when dry matter was weighed *directly* into the cultures instead of adding an aliquot from a wet digestion solution in which all organic matter had been destroyed. The possibility of toxicity due to traces of residual digestion acids could not be ruled out but the blank cultures showed no visual indications of toxicity or abnormal mycelium and in instances where molybdenum was introduced by the solution growth was normal. On the other hand the mycelia produced in cultures to which dry matter had been added direct were unusually uniform and pure white, often mainly on the surface of the fluid, whereas for the corresponding levels in growth series or digest aliquots the tendency was for a slightly grey tint and mucous or semi-mucous mycelium at low levels. The possibility that a beneficial organic growth factor or a more readily assimilated source of nitrogen was supplied which would have been destroyed during digestion merits consideration. The total available nitrogen supplied by 20 mg of dry matter would not be likely, however, to exert an appreciable effect on growth. So far these possibilities have not been distinguished or proved, but the assay of molybdenum in organic material evidently needs further investigation. As, however, the descrepancies were not usually great (table XIII) and fair agreement with chemical analyses was obtained the effect, though of interest, was not such as to invalidate the application of the method.

THE MOLYBDENUM STATUS OF SAND CULTURES

Water Supplies.

Tests were made on various types of water supplies available for use in the culture experiments. These included tap, distilled and demineralised water and the probable molybdenum concentrations are shown in tabel X. The effects of dithizone extraction of distilled water obtained from tinned copper stills with silver condensers (as compared with controls after *similar extraction* of twice glass distilled water) are given. The extent of contamination that may be introduced by manipulation of water supplies in relation to the molyb-

denum status of the culture solutions for which the water was used for dilution, and the order of molybdenum impurities in vaseline used for the large pyrex glass stopcocks are also indicated. All tests represent the *differences* between "nil" (2× glass distilled water) and

TABLE X

Examples of the Molybdenum Concentration in Water Supplies and Vaseline as Determined by the *A. niger* Method

Sample	Weight or Volume Taken	Yield Mycelium mg	Mo Estimated in Culture μg	Mo p.p.m. Material (Mean)
Once glass distilled water used exclusively for —Mo work	40 ml 40 ml 40 ml	78, 70, 87 92, 88, 41 32, 23	0.00009 0.00008 "nil"	0.0000022 0.0000020 "nil"
Similar water used for +Mo work	40 ml 40 ml	171, 131, 138 45, 95	0.00038 0.0004	0.0000094 0.00001
Water from tinned copper stills with silver condensers before extraction with purified dithizone solution	40 ml 40 ml 40 ml	51, 76, 100 45, 62, 58 16, 27	0.00008 0.00002 (almost nil)	0.000002 0.0000005 "nil" *)
Similar water after extraction	40 ml	628, 477, 471	0.00253	0.000063
Twice glass distilled water after extraction	40 ml	96, 75, 74	0.00011	0.0000023
Difference calculated	—	—	0.00242	0.000061
Water from tinned copper stills with pyrex condensers	40 ml 40 ml	58, 55, 44 48, 51	"nil" 0.0001	"nil" *) 0.0000025
Tap water from Silical Water Softeners	10 ml 40 ml 10 ml 10 ml	255, 157 844, 841, 879 267, 334, 340 726, 640	0.0013 0.0053 0.0011 0.0033	0.00013 0.00013 0.00011 0.00033
Rain water from storage tanks (*a*) (*b*) (*c*)	10 ml 10 ml 10 ml	179, 163 460, 420, 467 142, 245	0.009 0.0020 0.001	0.00009 0.0002 0.0001
Demineralised rain water after passage through 2 Zeocarb and 1 Deacidite E columns (*a*) (*b*) (*c*)	30 ml 40 ml 30 ml 30 ml	67, 57 476, 490, 481 486, 420, 467 147, 198 % removal by resin treatment = 72.5%—83%; Mean 76.4%	0.0004 0.0022 0.00147 0.0008	0.000015 0.000057 0.000048 0.000026
Vaseline untreated	1.025 g	897	0.01	> 0.01
Vaseline extracted by boiling in 3 separate lots of glass distilled water	1.025 g	137	0.0009	0.0009

*)These values here may be inaccurate due to masking effect noted for water from stills with silver condensers.

the test samples. Typical results are also illustrated in photograph no. 6.

Agreement between independent determinations is on the whole good and gives grounds for reasonable confidence. The masking of the true molybdenum status of the metal distilled water (from the silver stills) until a dithizone extraction had been performed is of interest for two reasons. First it suggests that the method may be liable to interference in some circumstances, and second that heavy metals (as yet unidentified but possibly tin, copper or silver) may "block" or antagonise the action of molybdenum at low concentrations in the metabolism of this organism, and possibly of others requiring this element.

The molybdenum concentration in all samples except from the glass stills (and possibly, but by no means, certainly, from the tinned copper stills with pyrex condensers) was in excess of that likely to interfere with the production of acute molybdenum deficiency effects. This conclusion is based on the results of unpublished work by Dr. S. C. Agarwala at Long Ashton where outstanding responses in cauliflower plants to as little as 0.00005 p.p.m. molybdenum have been recorded.

Sand.

As with soil, sand also contains available molybdenum and the amount of this fraction has been estimated in a number of samples by the *A. niger* method. Normally 2-10 g of sand were added prior to sterilisation. The results obtained after chemical analysis of the total acid soluble (hot 20% HCl) fraction using the method of Piper and Beckwith (1948) [24]) are also given for comparison. The ratio of the two fractions were usually of the order of 1 : 10 respectively. The data are given in tabel XI where it will be seen that the method allows the progressive removal of even minute concentrations of molybdenum to be followed during the course of sand purification. The actual details of the process have been described elsewhere by Hewitt (1947, 1951) [5]) [6]) and by Hewitt and Jones (1947, 1949, 1950, 1951) [7]) [8]) [9]) [10]).

Molybdenum available in Sand Cultures.

The possibility that the available molybdenum present in sand cultures may accumulate during the season was also tested. An estimate of the probable available molybdenum supply to plants grown in purified sand cul-

TABLE XI

Available and Total Acid Soluble Molybdenum in Samples of Sand Used for Culture Experiments

Sample	Wt. Taken g	Yield of Mycelium mg	μg Mo Estimated (Mean)	Mean Available Mo p.p.m.	Total Acid Soluble Mo p.p.m.
Leighton Buzzard (Bedfordshire)					
Double Archies					
Big Pits No. 21	0.25	88, 9[illegible], 129	0.000203	0.00081	—
— do —	2.0	202, 225, 230	0.00070	0.00035	—
— do —	2.0	461, 433	0.0022	0.0011	—
No. 21 Big Pit					
Exface	2.0	285, 381, 447	0.0018	0.0009	0.006
(retained 24 mesh)	2.0	435, 477, 512	0.0027	0.00135	0.012
(passed 24 mesh)	2.0	591, 661, 760	0.0057	0.00285	0.032
3/32 Screen Washed	2.0	576, 639, 757	0.0053	0.00265	0.024
No. 21 Big Pit Iron Stained	2.0	592, 708, 730	0.0056	0.0028	0.015
— do — Pink Stained	2.0	589, 629, 727	0.0054	0.0027	0.029
Front Pit Exface	2.0	236, 314, 349	0.0013	0.00065	not detected
Aylesbury Pit 60 Carbonate boiled	2.0	660, 679, 808	0.0071	0.00355	0.050
Aylesbury Pit 60 Raw Exface	2.0	547, 584, 694	0.0043	0.00215	0.047
Leziate Pits (Norfolk)					
Silica Sand No. 851 (washed)	2.0	829, 1154	0.01	0.005	
	5.0	675, 828, 853	0.0060	0.0012	0.043
As above (acid extracted)	2.0	209, 201	0.0013	0.00065	—
	10.0	298, 414, 436	0.0022	0.00022	—
Fine Sand No. 852 (washed)	5.0	681, 715, 767	0.0055	0.0011	0.019
As above (acid extracted)	10.0	690, 710, 781	0.0056	0.00056	—
Coarse Sand No. 853 (unwashed)	5.0	688, 698, 766	0.0055	0.0011	0.044
As above (acid extracted)	10.0	639, 701, 739	0.0045	0.00045	—
Loch Aline Pits (Scotland) *a*	2.0	302, 311, 374	0.0023	0.00115	0.02
Fraction passing 0.5 mm sieve *b*	2.0	699, 745, 776	0.0070	0.0035	—
Rakestadt (Norway)					
Quartz	2.0	598, 603, 669	0.0053	0.00265	—
Coarse 24–50 mesh	2.0	655, 720, 736	0.0072	0.0036	—
	2.0	792, 907	0.006	0.003	—
	2.0	1134, 1070	0.01	0.005	—

TABLE XI (continued).

Sample	Wt. taken g	Yield of Mycelium mg	μg Mo Estimated (Mean)	Mean Available Mo p.p.m.
Sand Purification				
Leighton Buzzard				
Initial	0.25	88, 92, 129	0.000203	0.0008
After 1st acid run	2.0	435, 413	0.0026	0.0013
unleached samples	2.0	523, 558, 908	0.00362	0.0018
leached sample	2.0	85, 122, 268	0.00044	0.00022
After 2nd acid run				
unleached sample	2.0	167, 333, 337	0.0010	0.0005
leached sample	2.0	65, 80, 96	0.00010	0.00005
After 3rd acid run				
unleached samples	2.0	68, 80, 112	0.000135	0.000067
leached sample	2.0	47, 50, 86	0.00006	0.00003
Sand leached in pots				
and ready for use	2.0	77, 122, 175	0.00015	0.000075
Sand treated 3 times				
with acid and once	10.0	19, 39, 42	0.00015	0.000015
with alkali	10.0	16, 21, 22	"nil"	below 0.000005
Leachate samples from pots before sowing	20 ml	for 8 pairs range 37–167 mg 12 between 40 and 80 mg mean 83 mg	0.00027	0.000013

tures was obtained at the same time. The results of some tests are shown in table XII. They indicate an appreciable increase in molybdenum content of leachates by the end of the season, due in part presumably to dust, and in part to decomposing roots.

THE MOLYBDENUM STATUS OF PLANT MATERIAL

The *A. niger* method has been applied to the determination of molybdenum in dry material when inadequate for ash analysis, in dissected regions of fresh plant tissues and in guttation fluid or vascular sap.

Dry Material.

Two methods as noted above have been tried.

They are (*a*), use of an aliquot after wet digestion of 100–250 mg of dry material and (*b*), addition of 10 50 (usually 20.0) mg of dry material direct to the cultures before sterilisation. Results have been compared with data obtained by orthodox chemical analysis using the method of Piper and Beckwith (1948) [24].

TABLE XII

Molybdenum of Leachates from Molybdenum Deficient Sand Culture in Pyrex Glass Pots 4 weeks after Commencement of Work				
Sample	Volume Used	Yield of Mycelium mg	(Sand treated with 3 acid runs) Mo Estimated μg	Mo p.p.m. in Leachate
Leachate from Cauliflower culture I	10 ml	56, 63	0.00004	0.000004
,, ,, II	10 ml	56, 73	0.000045	0.0000045
Rape ,,	10 ml	55, 60	0.000025	0.0000025
Savoy Cabbage ,,	10 ml	61, 89	0.00008	0.000008
Swede ,,	10 ml	56, 71	0.000045	0.0000045
Kale (Marrowstem) ,,	10 ml	80, 89	0.00012	0.000012
Kale (Hungrygap) ,,	10 ml	86, 103	0.00015	0.000015
Sprout ,,	10 ml	55, 60	0.000025	0.0000025
Leachate from —Mo pots at end of season (5 months)	10 ml	120, 161	0.00045	0.000045

Procedure: Ash analysis of 100–250 mg dry material was carried out by adding 2—3 ml redistilled nitric acid (Judactan Batch Analysed Grade) in a 50 ml pyrex conical flask and then 0.2 ml 60% perchloric acid after partial decomposition on a hot plate. The digested residue was evaporated to dryness and 10 ml. water added. This was again evaporated to secure complete removal of all residual acids. The residue after heating with water was made up to 50 ml in the same flask (already calibrated) and 5 ml aliquots were taken for each determination. Roots were treated similarly except that 200–500 mg of dry material was used and the weight of sand (if any) determined on the evaporated residue (after completion of the analysis), from which the true dry weight was calculated.

Dry material for digestion or direct addition to cultures was ground to fine powder with a pyrex glass pestle and mortar and weighed by difference from a clean dry miniature glass beaker of 5 ml capacity.

Results: The results shown in tabel XIII were obtained with a range of molybdenum deficient plants grown in sand culture. They indicate that the order of molybdenum content in dry plant material can be determined with reasonable accuracy considering the extremely minute concentration present. The economy in material is so great that the method seems amply justified and replication of determinations is rendered possible. In many instances there is no satisfactory alternative at present as the chemical method requires 1–10 g of molybdenum deficient material for analysis. A chemical method applicable to 0.1–0.5. μg molybdenum would be of great value however for this type of work as sufficient dry material is usually available.

TABLE XIII

Determination of Molybdenum in Deficient Plant Dry Material by Different Methods

Molybdenum Deficient Plant	Mo p.p.m. in Dry Material Determined		Mean Value of two Cultures
	Digestion method	Direct Method	Chemical Method
Savoy cabbage			
Young leaf (I)	0.219		0.28
(II)	0.113		0.051
Old leaf	0.076		
Stem	0.090	0.0[illegible]2	0.10
Root (I)	0.076		
(II)	0.072		
Rape			
All tops	0.083		
Old leaf	0.103		
Marrowstem kale			
Young leaf (I)	0.046	0.015	0.069
(II)	0.01		
Old leaf (I)	0.206	0.124	0.077
(II)	0.125	0.132	0.073
(III)	0.064		
Stem	0.044	"nil"	0.049
Root (I)	0.121		
(II)	0.070		
Hungrygap kale			
Young leaf	0.030		
	0.025		
Old leaf (I)	0.050		0.02
(II)	0.099		
Stem	0.174		
Root	0.017		
Cauliflower			
Young leaf (I)	0.025		
(II)	0.066		0.13
(III)	0.110		
Old leaf	0.040	0.0[illegible]0	0.09[illegible]
Stem	0.051	0.0[illegible]1	0.04[illegible]
Root	"nil"		
Brussels sprout			
All tops	0.084		

Digestion Blanks: 33, 44, 47, 86 mg. Mean 52.5 mg = 0.00003 μg approx.
Range of Mo determined: "nil" to .003 μg.

Fresh Material.

The molybdenum content of normal plants is generally between 0.2 and 1.0 p.p.m. in fresh weight, i.e. .002–.01 μg per 10 mg. This range conveniently allows the method to be used for the study of molybdenum distribution on a histological scale. So far little has been done in this direction but the results below were obtained with 2–4 mg of fresh tissue.

Vascular bundle of petiole:	Wt. (approx.)	μg Mo found	p.p.m.
for molybdenum deficient plant *)	3.5 mg	0.00045	0.13
,, ,, excess plant	2.5 mg	0.01	4
Cortex of peticle:			
for molybdenum deficient plant *)	3.5 mg	0.00015	0.043
,, ,, excess plant	2.5 mg	0.004	1.6

*) Molybdenum levels in culture were 0.0005 and 0.5 p.p.m. respectively.

Molybdenum in guttation fluid.

Occasionally, on cool, humid mornings considerable guttation from leaf margins or cut petioles occurs. It was thought of interest to determine the molybdenum concentration of this fluid in relation to the concentration in dry matter and in the nutrient solution used. The method used was as follows. U shaped capillary tubes with one limb about 5 cm and the other about 3 cm long with a flared top were made, cleaned, dried and weighed. The guttation droplets attached to leaf margins were collected in the flared opening. The tubes were then carried horizontally in clean petri dishes and reweighed immediately. The total weight collected was between 4 and 95 mg mainly according to expected molybdenum status. The fluid was washed out of the tubes into the culture flasks by careful addition of droplets of water from a wash bottle and inverting the tube between each flushing. Table XIV shows some results obtained in September 1949. Appreciable concentrations of molybdenum only escaped when excessive levels were given in the nutrient.

DISCUSSION

The results presented in the first part covered general technique, the purity of materials and the elimination of traces of molybdenum. They showed that attention to detail is of the utmost importance in obtaining consistent results and low yields for "nil" and "low molybdenum" cultures.

It is evident that *A. niger* can be made to respond to as little as 0.0001 μg or even 0.00005 μg molybdenum when care is taken to eliminate extraneous contamination. The molybdenum concentration in twice glass distilled water is low enough to meet these exacting requirements. Capacity of culture flask, spore inoculum and incubation all exert small effects on the nature of the response. Nutrient reagents, however, present the most significant source of contamination.

Simple and efficient methods are available for the purification of nutrient reagents. The several methods tested were found, however, to differ greatly in their efficiency. Co-precipitation with copper sul-

TABLE XIV

Molybdenum Found in Leaf Guttation Fluid and in Dry Leaf Material

Plant	Nutrient Status	Source of Guttation	Wt. of Fluid Used	Yield of Mycelium mg	μg Mo Estimated	p.p.m. in Fluid	p.p.m. in Dry Matter
Cauliflower	Mo deficient						
	low Mn	leaf margin	95.2	30	—	below 0.001	0.05–0.25
	normal Mn	,, ,,	65.7	24	—	0.002	0.02–0.05
	low Mn	cut petiole	87.0	39	—	0.002	—
	normal Mn	,, ,,	49.7	93	0.00015	0.003	—
	Normal Mo 0.02 p.p.m.						
	normal Mn	leaf margin	23.4	38	—	below 0.005	1.6
	low Mn	cut petiole	16.6	30	—	0.005	—
	high Mn	,, ,,	7.7	59	0.0001	0.015	0.6
	Excess Mo 9.6 p.p.m.						
	normal Mn	cut petiole	37.7	1010	0.02	0.55 or above	470
	high Mn	,, ,,	4.5	900	0.01	2.2	
	high Mn	leaf margin	6.8	975	0.015	2.2	1500
Tomato	Normal Mo 0.02 p.p.m.						
	normal Mo	leaf margin	22.8	49	0.00005	0.002 approx.	2 3 approx.
	excess Mo 9.6 p.p.m.	,, ,,	16.6	1080	.02	1.2	—
Blank Determination				41 mg			

phide or mixed sulphides appears to be the most efficient, simple and consistently reliable procedure. The quantitative efficiency readily attains 99.5% or may exceed 99.95% but the initial molybdenum concentration and the concentration of collecting metal used to form the sulphide, control the actual efficiency achieved. The importance of these apparently small differences lies in the fact that the *residual* concentration in one method may be 10–50 times that in another and the effect of these differences is readily detectable.

In the light of the results obtained in this work a method recommended by Nicholas and Fielding (1950) [20]) does not seem justified. In this method 10 μg Mo were added per 200 ml concentrated culture media *prior* to purification by mixed copper and lead sulphide precipitation at pH 3.0. The experiments reported here indicate clearly that the removal of molybdenum although highly efficient is probably in the nature of an equilibrium reaction except possibly at the lowest concentrations tested. Addition of a known amount of molybdenum leads to the retention of a proportion in the filtrate. That co-precipitation reactions may behave according to an adsorption isotherm has been shown recently by Burriel-Marti and Caldas (1950) [2]) for the co-precipitation of vanadium by hydroxides of iron, aluminium or chromium. For critical work a double co-precipitation is considered the most reliable procedure.

Cadmium sulphide alone appears relatively inefficient. The specificity of metal sulphides as collectors in co-precipitation reactions is discussed by Feigl (1949) [3]) (pp. 163–164 and 467–468) especially, and also by Sandell 1944) [25]). Feigl [3]) (p. 164) points out that copper is especially satisfactory as a collector for zinc, lead and molybdenum, whilst mercury is especially effective as a collector for lead but not for silver or copper. The experience recorded in this work is thus in harmony with the data given by Feigl.

Feigl (1949) [3]) (*loc. cit.*) also stated that colloidal sulphur liberated by the reaction between hydrogen sulphide and a trace of iodine greatly increased the efficiency with which molybdenum sulphide could be removed by precipitation. Under these conditions the precipitation was said not only to be practically complete but also coagulated and readily separated by filtration, a result achieved by hydrogen sulphide alone only when used under pressure.

The results obtained here did not indicate that the addition of

iodine in any way improved the efficiency with which molybdenum was removed, and the use of iodine and hydrogen sulphide alone was useless. Nicholas and Fielding (1951) [21]) recommended the addition of iodine when sulphide precipitation was used. As iodine or iodide may be toxic to organism its inclusion does not seem entirely justified.

The use of 8-hydroxyquinoline and iron first used in work of this nature by Hewitt and Jones (1947, 1949) [7]) [8]) was also shown to be efficient and confirmation of this was reported by Nicholas and Fielding (1950) [20]). It was found, however, that copper or certain other sulphide precipitation methods were slightly more efficient than 8 hydroxyquinoline. On the other hand use of the latter may present advantages on occasions. Thus several other microelements including copper, zinc, nickel, cobalt and gallium may be removed at the same time and there is less likelihood of introducing copper should a solution free from this or all microelements be required. The methods of Scott and Mitchell (1943) [26]) and Mitchell and Scott (1948) [14]) on which the latter technique is based offer great scope in this field and further studies on the production of other micro-element deficiencies by such means are in progress.

Recrystallisation was shown to be not only inefficient but also inconsistent in effect. Potassium nitrate only was efficiently purified by this means. An important point was the apparent increase in contamination shown by phosphate salts after recrystallisation. It is possible that traces of molybdenum were carried down during crystallisation thus effecting a concentration. This might occur if a phospho-molybdate complex were formed. The low efficiency of the early alkaline earth phosphate adsorption technique of Steinberg (1935) [27]) and Stout and Arnon (1939) [31]), used by Steinberg (1936) [28]) *et seq.* and Arnon and Stout (1939) [1]) in their molybdenum deficiency studies, was demonstrated. This confirmed the conclusion reached by the writer in earlier unpublished work, by Piper (1940, 1944) [22]) [23]) and by Hewitt and Jones (1947) [7]). Such removal as may be achieved is probably based on the formation of a phospho-molybdate complex.

The various types of methods examined here thus differed from each other in their approximate efficiency by a considerable margin. The precise value of the efficiency of a given method also varied

slightly from one trial to another, but the different methods were consistent in their relative efficiency. The causes of the variation in absolute efficiency were not determined. It is possible that minor differences in temperature or pH, uncertainty as to exact concentration of molybdenum contamination when inferred from results with very low concentrations, or other factors may have been responsible and further investigation is needed to elucidate this point.

The favourable comparison between the efficiency of demineralisation and of distillation for water purification is especially interesting. The equilibrium between adsorbed and free molybdenum ions may exceed 99.5% in favour of the former, in spite of their relatively weak dissociation, when suitable resins are used. The application of the ion-exchange resins for large scale purification of water suitable for molybdenum deficiency studies with higher plants is thus possible and should greatly extend the scope of such experiments since preparation of pure nutrients on a comparable scale no longer presents any problem. The use of resins for the purification of sugars is also efficient. The method might be of value with other metabolites for which the use of copper or other metals was undesirable or impracticable.

The data presented in this paper show that the *Aspergillus niger* method can be used to detect and estimate with reasonable confidence, molybdenum in amounts of the order of 0.0001 or less to 0.01 (0.02) microgram.

25°C appears to be a more satisfactory temperature for incubation than 30°–32°C used by earlier workers as the range at the lower temperature is slightly extended for higher concentrations with improved sensitivity, although the gradient of the yield curve appears to be reduced at the lower end of the scale. The relation of onset of sporulation to maximum yield requires further study. The impression formed in this work is that profuse sporulation is often associated with slightly reduced yields. The results obtained by Löhnis (1944) [11]) are consistent with this possibility. It is reasonable to suppose that when spore formation begins, profound changes in metabolism may occur which perhaps result in a nett loss of assimilated carbon. The biochemical aspects of the role of molybdenum in the nutrition of *A. niger*, more especially as they concern nitrogen metabolism, will be the subject of a separate publication.

Experience shows that a single calibration curve based on many

separate determinations cannot be used unless a great many replicates are used for each assay. More reliable results are obtained by preparing a standard growth series for each occasion. Standard series showing abnormal values (high at the "nil" end, or low at the upper limit of the range) should be rejected and the determinations repeated. The cause of marked variation between mean values obtained on successive occasions is not understood. Imperfect control of inoculation, nutrient status, age and viability of the spores, lag in germination, or aeration of the culture media are possible factors requiring further investigation. Spore counts did not, however, bear any consistent relationship to this variability. The disappearance of nitrate from the culture fluid after incubation with 0.005 μg Mo or more suggests that nitrogen may have been partially limiting in some instances for rapid growth above this concentration of molybdenum and this point will be tested in future work. The variation in germination of spores was studied by McCallan and Wilcoxon (1939) [13]) in an extensive series of experiments with *Sclerotinia fruticola* where such factors as humidity, gas exchange, spore count, origin of inoculum, size of culture vessel, means of collecting spores, age of cultures, temperature, etc. were tested. Many of these factors had little or no consistent effect in variability but it was concluded that variation was reduced if spores were rubbed off the agar slopes in distilled water, filtered, washed and centrifuged to remove indeterminate amounts of stimulating materials derived from the agar. Such a procedure would be difficult in the work described here and in spite of this precaution McCallan and Wilcoxon (1939) [13]) concluded that day to day variation still exceeded that expected from a random sampling. The remedy suggested was, as adopted in this work, to make strict comparison only between experiments (or results) performed on the same day with similar reagents, inoculum and incubator conditions.

The accuracy of the method for assay purposes depends upon three main factors. (*a*) The precision in measuring aliquots and yields. This is the same as for a normal chemical procedure, and the fraction of error due to such factors is generally small. (*b*) Extraneous contamination by molybdenum extra to that required to be determined. This is the most serious cause of error and lack of sensitivity. Use of the standard growth series prepared in identical fashion eliminates part of the error by difference but at the expense

of sensitivity. Contamination not uniformally distributed, i.e. that due to dirty flasks, pipettes or stray particles or splash (as opposed to that due to the water and bulk nutrient) cannot be compensated. With care and experience gained in routine manipulation this factor is not usually above 0.00005 to 0.0001 microgram. (*c*) Temporally uncontrollable variation already mentioned. This is partly compensated by the use of standard growth series each time. Its existence implies, however, that such variation may also affect replicate cultures as well as between results separated in time. Threefold replication appears to be a reasonably satisfactory safeguard against excessive error due to this factor.

Much of the error due to (*b*) and (*c*) might be eliminated by use of a two point assay technique. The effect of unknown constituents of the test material on the response of the fungus for limited amounts of molybdenum also needs more study. The results of removing unidentified heavy metals for the distilled water samples shows that heavy metal antagonism may mask the presence of small amounts of molybdenum. There may be other examples of such interference leading to low results. The introduction of organic or other partly assimilated nutrients when organic materials are assayed for molybdenum may possibly cause interference if the molybdenum requirement is reduced thereby and this would lead to high results. These aspects of the work will be dealt with in a subsequent paper.

The most successful results from the use of the *A. niger* method depend on the best compromise between the increased sensitivity shown at lower levels and the reduction of error due to contamination by assay of relatively high concentrations. The application of this principle suggests that the limits of molybdenum in the sample should lie between 0.0003 and 0.0075 μg. The 25 fold range is, however, still comparable with that of chemical methods but at a lower absolute concentration. When necessary some extension of the range is possible but at the expense of accuracy or sensitivity.

The assays made on plant material indicate the promising application of the method in this direction. The determination of molybdenum in samples of dried material too small for analysis by chemical means is but one aspect of the greatly extended scope of such studies. It was feasible, as shown above, to determine molybdenum in different histological regions with samples weighing only a few milligrams (or possibly less for plants given adequate concentra-

tions). The estimation of molybdenum in guttation fluid and sap from cut surfaces should also lead to interesting results in relation to the factors determining the permeability of tissues to the element, and the relation of uptake to translocation, accumulation and exudation.

The molybdenum concentrations determined in sand, water samples and leachates indicate that the probable range of contamination is quite wide. The raw materials commonly used in culture experiments were all found to contain appreciable amounts of the element, when tested by the method given here, and such small points as the use of vaseline for stopcocks require attention. Suitable purification methods eliminated the molybdenum contamination in sand and water by progressive stages until satisfactory low levels were attained. The method was well suited to following the progress of elimination during these procedures and for keeping a "check" on routine samples before use in the culture experiments. In the recent work of Hewitt and Jones (1950, 1951) [9] [10] sand, water and nutrient reagents have been tested as indicated. The method has been a safeguard against possible unsatisfactory preparative work. The assurance gained is well worth the slight delay involved, since in the event of faulty materials the cause even of repeated failure might not be ascertained without tedious and extensive work.

SUMMARY

1. This paper comprises a study of some of the factors concerned in the detection and removal of molybdenum present in culture materials and its assay in these and plant materials by means of *Aspergillus niger*.

2. Nutrient reagents are one of the main sources of irregular contamination by molybdenum and may contain more than 0.1 or less than 0.001 p.p.m. of the element.

3. Purification methods tested included recrystallisation, the alkaline phosphate adsorption, coprecipitation by 8 hydroxyquinoline and coprecipitation by single or mixed sulphides, sometimes in the presence of iodine or combinations of these methods.

4. Sulphide coprecipitation was generally the most efficient and reliable. Copper and lead precipitated by H_2S were usually the best collectors, arsenic was also good and silver and mercury were fair. Cadmium alone or sulphides precipitated by ammonium sulphide were poor. Mixed sulphide precipitation was also highly efficient. The presence of iodine did not appreciably enhance the effectiveness of the procedure except when high pH values were necessary and was useless alone.

5. Quantitative values for efficiency of removal were determined and

were of the order of 30% for phosphate adsorption, nil to 90% for recrystallisation according to salt, 92–98% for 8 hydroxyquinoline and 98–99.95% for sulphide precipitates except cadmium (84%).

6. The effect of different copper and molybdenum concentrations were also tested. Addition of molybdenum above about 0.1–0.5 μg per 50 ml of medium not only caused increased residual molybdenum but reduced the per cent removed. There was a marked improvement with increase of copper concentration from 25 p.p.m. up to 5000 p.p.m.

7. For routine work a single or double sulphide coprecipitation with copper at 500 p.p.m. was employed with reagents already recrystallised twice with addition of alcohol. Twice glass distilled water and rigorously cleaned hysil or pyrex glass were more satisfactory.

8. Yields of mycelium in the absence of added molybdenum were usually about 35–40 mg. Values as low as 15–19 mg and up to 96 mg were recorded. Yields in the presence of 0.01 μg attained 1000 mg but were usually about 850 mg. Temperature of incubation (usually 25°C) and other factors caused variation in the range recorded on any occasion. The curve relating yield to log Mo concentration was sigmoid between 0.0001 and 0.02 μg.

9. Determinations were made in duplicate or triplicate cultures with a duplicate calibration (at 26°C) series for yields in the presence of graded molybdenum levels from "nil" to 0.01 μg. Fair agreement was normally obtained.

10. Points noted in addition to the study of nutrient purification methods were the use of ion exchange resins for water and sugar treatments, the contamination and purification of silica sands and water supplies. The available molybdenum present in a sand culture and the molybdenum status of deficient plant material and of leaf exudates was also investigated in some detail.

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REFERENCES

1) Arnon, D. I. and Stout, P. R., Molybdenum as an essential element for higher plants. Plant Physiol. **14**, 599 (1939).
2) Burriel-Marti, F. and Caldas, E. F., Phenomena of coprecipitation in analysis. I. Coprecipitation of vanadium with iron, chromium and aluminium hydroxides. Anal. real. Soc. Csp. Fis. Quim., **46** B 37. Courtecy British Abstracts C. 318 (1950).
3) Feigl, F., Selective sensitive and specific reactions. Academic Press. Transl. by R. E. Oespur. New York (1949).
4) Gerretsen, F. C., On the use of *Aspergillus niger* for the determination of plant nutrients in the soil. Anal. Chim. Acta. **2**, 781 (1948).
5) Hewitt, E. J., A technique for large scale pot sand cultures. Ann. Rep. Long Ashton Res. Sta. for 1946, 37 (1947).
6) Hewitt, E. J., The use of sand and water culture methods in the study of plant

nutrition. A Technical Communication to be published by the Commonwealth Bureau of Horticulture and Plantation Crops. In the press (1951).

7) Hewitt, E. J. and Jones, E. W., The production of molybdenum deficiency in plants in sand culture with special reference to tomato and *Brassica* crops. J. Pomology and Hort. Sci. **23**, 254 (1947).

8) Hewitt, E. J. and Jones, E. W., Molybdenum as a plant nutrient. The effects of molybdenum deficiency on some vegetables, cereals and forage crops. Ann. Rep. Long Ashton Res. Sta. for 1948, 81 (1949).

9) Hewitt, E. J. and Jones, E. W., Molybdenum as a plant nutrient. II. Effect of molybdenum deficiency on some *Brassica* crops. Ann. Rep. Long Ashton Res. Sta. for 1949, 58 (1950).

10) Hewitt, E. J. and Jones, E. W., Molybdenum as a plant nutrient I. The influence of molybdenum on the growth of some *Brassica* crops in sand culture. J.Hort. Sci. In the Press (1951).

11) Löhnis, M. P., The action of manganese on the development of *Aspergillus niger*. Antonie van Leeuwenhoek **10**, 101 (1944).

12) McCallan, S. E. A. and Wilcoxon, F., The action of fungous spores on Bordeaux mixture. Contrib. Boyce Thompson Inst. **8**, 151 (1936).

13) McCallan, S. E. A. and Wilcoxon, F., An analysis of factors causing variation in spore germination tests of fungicides. I. Methods of obtaining spores. Contrib. Boyce Thompson Inst. **11**, 5 (1939).

14) Mitchell, R. L. and Scott, R. O., Concentration methods in spectrographic analysis. II. Recovery of these constituents in plant material and soil extracts by mixed organic reagents. J. Soc. Chem. Ind. **66**, 330 (1948).

15) Mulder, E. G., On the use of micro organisms in measuring deficiency of copper and molybdenum in soils. Antonie van Leeuwenhoek **6**, 99 (1939).

16) Mulder, E. G., Importance of molybdenum in the nitrogen metabolism of microorganisms and higher plants. Plant and Soil **1**, 94 (1948).

17) Mulder, E. G., The microbiological estimation of copper, magnesium and molybdenum in soil and plant material. Anal. Chim. Acta. **2**, 793 (1948).

18) Nicholas, D. J. D., Use of *Aspergillus niger* for determining magnesium, zinc, copper, molybdenum and manganese in soils and plants. Fertilizer Soc. Proc. 13 (1950).

19) Nicholas, D. J. D. and Fielding, A. H., The use of *Aspergillus niger* (M strain) in the bioassay of magnesium, copper, zinc and molybdenum in soil. Ann. Rep. Long Ashton Res. Sta. for 1947, 126 (1948).

20) Nicholas, D. J. D. and Fielding, A. H., Use of *Aspergillus niger* as a test organism for determining molybdenum available in soils to crop plants. Nature **166**, 342 (1950).

21) Nicholas, D. J. D. and Fielding, A. H., The use of *Aspergillus niger* (M) for the determination of magnesium, zinc, copper and molybdenum available in soils to crop plants. J. Hort. Sci. **26**, 125 (1951).

22) Piper, C. S., Molybdenum as an essential element for plant growh. J. Australian Inst. Agr. Sci. **6**, 162 (1940).

23) Piper, C. S., Manganese deficiency in oats. Nature **153**, 197 (1944).

24) Piper, C. S. and Beckwith, R. S., A new method for determination of small amounts of molybdenum in plants. J. Soc. Chem. Ind. **67**, 374 (1948).

25) Sandell, E. N., Colorimetric determinations of traces of metals. Interscience New York (1944).

26) Scott, R. O. and Mitchell, R. L., Concentration methods in spectrographic analysis. I. Recovery of cobalt, nickel, molybdenum, copper and zinc in plant materials and soil extracts by 8 hydroxyquinoline. J. Soc. Chem. Ind. **62**, 5 (1943).

27) Steinberg, R. A., Nutrient solution purification for removal of heavy metals in deficiency investigations with *Aspergillus niger*. J. Agr. Research **51**, 413 (1935).
28) Steinberg, R. A., Relation of accessory growth substances to heavy metals including molybdenum in the nutrition of *Aspergillus niger*. J. Agr. Research **52**, 439 (1936).
29) Steinberg, R. A., Role of molybdenum in the utilisation of ammonium and nitrate nitrogen by *Aspergillus niger*. J. Agr. Research **55**, 891 (1937).
30) Steinberg, R. A., Effects of nitrogen compounds and trace elements on growth of *Aspergillus niger*. J. Agr. Research **59**, 731 (1939).
31) Stout, P. R. and Arnon, D. I., Experimental methods for the study of the role of copper, manganese and zinc in the nutrition of higher plants. Am. J. Botany **26**, 144 (1939).
32) Stout, P. R. and Meagher, W. R., Studies of the molybdenum nutrition of plants with radiactive molybdenum. Science **108**, 47 (1948).
33) Welcher, F. J., Organic analytical reagents (volumes 1–4). Van Nostrand, New York (1947).

ERRATUM

Vol. III, p. 176, footnote:

for $S(x - M)^2/N - 1$ read $\sqrt{S(x - M)^2/N - 1}$.